PRACTICAL PHYSICAL
AND
COLLOID CHEMISTRY
L. MICHAELIS

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Practical Physical and Colloid Chemistry

For Students of Medicine and Biology

BY

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Authorised Translation from
the Second German Edition

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TRANSLATOR'S PREFACE.

In the preface to the first edition of this book Professor Michaelis laments the neglect of the study of Physical Chemistry among the Physicians and Biologists of his own country; but in the preface to the second edition, which appeared less than two years later, he rejoices at a sudden awakening of interest in the subject. Here in England we are not accustomed to such violent reversions of opinion, but we do find a steadily increasing demand for training in those aspects of Physical and Colloid Chemistry that are of importance for the study of Biology and Medicine, and it is with the object of meeting in some measure this demand that I have prepared the present translation of Professor Michaelis' work. The carefully chosen set of practical exercises here presented, interspersed with succinct but clear theoretical explanations, seemed to me to be so valuable that it would be regrettable if any English readers were debarred from access to them on account of difficulties of language.

In translating a work into one's own language one is inevitably brought face to face with the question of the utility of learning foreign languages for scientific purposes; and I venture to express the hope that the existence of the present work in an English translation will in no way deter the student—and particularly the young student—from acquiring facility in the language in which it was originally written. For no scientific training can be regarded as in any way complete without an adequate working knowledge of the chief languages in which scientific work is published; for although outstanding works may become available in translations, the original articles to which reference is made—and these constitute the bulk of scientific literature—are accessible only to those who can consult them in the original.

And further—anyone who struggles painfully with a scientific

article in a language other than his own is helping to achieve that spirit of international co-operation which forms not the least of the rewards of scientific endeavour; he is assisting to raise scientific culture to a position of independence of national boundaries, and in some measure to ensure to it its rightful domain, in which political barriers between peoples are unknown and the rivalries of the leaders of nations are not heard.

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September, 1925.

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I.

The Principle of Serial Experiments.

A type of problem we shall frequently meet in the application of physical chemistry to biological investigations consists in the determination of the quantity of some active material required to produce some particular effect; such as, for example, the quantity of sodium chloride that is necessary for the complete precipitation of a colloid; or the quantity of a hæmolysin that brings about immediate and complete hæmolysis in a blood solution of a given composition; or the optimum concentration of hydrogen ions for the precipitation of egg-white; or the amount of an indicator which produces a particular depth of colour in a solution. One of the commonest cases arising in practice is the estimation of the quantity of serum from a sensitised rabbit required for the immediate and complete haemolysis of a given amount of sheep's blood-corpuscles suspended in a given volume of solution containing a given concentration of complement. First of all we make a rough trial with 1 c.c., ·1 c.c., ·01 c.c., ·001 c.c. and so on, and discover that the volume of serum required for immediate hæmolysis is between, say, ·001 and ·0001 c.c. Let us, then, carry out a series of more delicate tests with—

Nr. 1	2	3	. . . .	8	9	10
·0001;	·0002;	·0003;	. . . .	·0008;	·0009;	·001 c.c.

Suppose that the blood is completely laked in the second tube but not in the first, we can say that the dose required for hæmolysis is ·0002 c.c. and that ·0001 c.c. is certainly too small. But we are not justified in excluding as insufficient any quantity larger than 50 per cent. of the value we have found. If we had found complete laking in tube No. 8 the hæmolysing dose would have been ·0008 c.c., and we should have known ·0007 c.c., that is a volume $^1/_8$ or $12^1/_2$ per cent. smaller, to be insufficient. The degree of exactness with which we can state our result thus depends on the actual value which the limiting dose may chance to possess. This is an unsound principle and an unsystematic method of work. If we begin the series with ·0001 c.c., ·0002 c.c., we should continue with ·0004 c.c.; ·0008 c.c. ·0016 c.c.—a geometrical series with the

factor 2. If we wish for finer gradations we use a geometrical series with the factor 1·5 or, for more delicate work still, 1·2, according to the accuracy ordinarily obtainable with the particular material.

Limiting ourselves to two significant figures, we obtain respectively in these cases such series as:

·0010 ·0015 ·0022

and

·0010 ·0012 ·0014 ·0017 ·0020

This particular interval is thus covered by three terms of a geometrical series having the factor 1·5, or by five terms if the factor is 1·2. For the interval from ·0060 and ·012 the corresponding series are, respectively:

·0060 ·0090 ·015

·0060 ·0072 ·0086 ·0103 ·0123

In dealing with a completely unknown material we should usually obtain a preliminary orientation using a series 1, 10, 100, etc., then investigate the appropriate range with a series with factor 2, and finally employ a more gradual series according to the nature of the material.

In cases where we need to cover a whole power of 10 the following series can be used:[1]

Each series covers the interval from ·1 to 1·0.

Factor of the series.	No. of terms.	Actual terms of series.
$\sqrt[2]{10}=3{\cdot}612$	3	·10 ·36 1·00
$\sqrt[3]{10}=2{\cdot}154$	4	·10 ·22 ·46 1·00
$\sqrt[4]{10}=1{\cdot}778$	5	·10 ·18 ·32 ·56 1·00
$\sqrt[5]{10}=1{\cdot}585$	6	·10 ·16 ·25 ·40 ·63 1·00
$\sqrt[6]{10}=1{\cdot}468$	7	·10 ·15 ·21 ·32 ·46 ·68 1·00
$\sqrt[7]{10}=1{\cdot}390$	8	·10 ·14 ·19 ·27 ·37 ·52 ·72 1·00
$\sqrt[8]{10}=1{\cdot}334$	9	·10 ·13 ·18 ·24 ·32 ·42 ·56 ·75 1·00
$\sqrt[9]{10}=1{\cdot}291$	10	·10 ·13 ·17 ·21 ·28 ·36 ·46 ·60 ·77 1·00

[1] E. Fuld, *Klin-therapeut. Wochenschrift* 1907, No. 11.

More frequently it happens that we wish to produce more gradual series by inserting extra terms into a rougher series with the factor 2. The terms of such series must possess the following ratios:

Factor of the series.	No. of terms.	Actual terms of series.						
2	2	1·00	2·00					
$\sqrt[2]{2}=1{\cdot}414$	3	1·00	1·41	2·00				
$\sqrt[3]{2}=1{\cdot}260$	4	1·00	1·26	1·59	2·00			
$\sqrt[4]{2}=1{\cdot}189$	5	1·00	1·19	1·42	1·69	2·00		
$\sqrt[5]{2}=1{\cdot}149$	6	1·00	1·15	1·26	1·41	1·59	1·78	2·00

Of course, it is not necessary to restrict oneself entirely to the use of these particular series. In the work that follows we shall use mostly arbitrary geometrical series with factors of various magnitudes.

The following two exercises are intended simply as illustrations of the practical application of these principles.

EXERCISE 1.

Quantitative Estimation of Rennin in Gastric Juice.[1]

In this case we can make only a quantitative comparison with an arbitrarily chosen standard ferment solution which can be made in the following way:

5 gm. of Grübler's pepsin are treated with 50 c.c. of 10 per cent. sodium chloride solution and allowed to stand for 8 days with occasional shaking. The mixture is then filtered, and to the filtrate an equal volume of glycerine is added. The resulting solution should keep indefinitely if stored in a closed vessel in a dark cool place. Naturally its exact strength will be a matter of chance: there is no generally-accessible and readily-reproducible rennin standard, each worker up to the present having set up his own.

The best test material is boiled milk to which has been added $^1/_{10}$ of its volume of 10 per cent. calcium chloride solution (calculated as the crystalline salt containing water; the exact strength is of little consequence).

[1] L. Michaelis and T. Rothstein. *Biochem-Zeitschr*, **105**, 60, 1920.

It is recommended to use as standard such a dilution of the rennin solution as will produce clotting in an equal volume of the calcium chloride-milk mixture in not less than 8–10 minutes at room temperature. This usually means diluting the original solution of Grübler's pepsin 10,000 times. We will call this dilution a solution of unit strength with regard to rennin; each c.c. shall be regarded as containing one unit of ferment (U.F.).

The gastric juice whose rennin content is to be estimated is first filtered; a series of dilutions are then made of it, ranging from the undiluted material to a 1 in about 500 mixture. (For juice of normal acidity a ten-fold dilution is the highest concentration that is required, but in cases of achlorhydria it is necessary to begin with the undiluted juice.)

A series of 10 test-tubes is taken and 1 c.c. of distilled water is placed in each except the first. Into this first tube 1 c.c. of gastric juice is measured by means of a 1 c.c. pipette that has been calibrated by blowing out. To the second tube (which already contains 1 c.c. of water) a c.c. of gastric juice is similarly added, the fluids are thoroughly mixed by repeated sucking up into the pipette, and then 1 c.c. of the mixture is transferred to tube No. 3, from which, after similar mixing, 1 c.c. is transferred to the next tube, and so on. When the 10th tube is reached, the last c.c. of fluid removed from this tube is rejected, so that the tubes each contain 1 c.c. of fluid consisting of the original gastric juice diluted in following proportions respectively:

$$^1/_1 \quad ^1/_2 \quad ^1/_4 \quad ^1/_8 \quad ^1/_{16} \quad ^1/_{32} \quad ^1/_{64} \quad ^1/_{128} \quad ^1/_{256} \quad ^1/_{512}$$

A further tube, specially marked, is now placed in the rack at about the middle position in the series; into it 1 c.c. of the unit rennin solution is measured. Then to each tube in turn 1 c.c. of the calcium chloride-milk mixture is added as quickly as possible from a 10 c.c. graduated pipette. Hardly one minute is required for the whole operation. Having regard to the roughness of the experiment, the time at which the filling is complete may be taken as the starting point; this is noted. The whole rack of tubes is now taken in the hand in order that their contents may be observed, the way in which the milk flows off the glass walls when the tubes are tilted being particularly noticed. It will soon be seen that the liquid in the first tube has become curdy. The object is to determine in which tube the fluid becomes curdy simultaneously

with the "control." Suppose, for example, that the contents of the control tube clot a little later than those of tube 7, but some considerable time before those of tube 8. A more accurate series of tests is then made using a new dilution of the gastric juice. Since the true value lies between $^1/_{64}$ and $^1/_{128}$, and nearer the former than the latter value, a dilution of gastric juice of $^1/_{50}$ is made, and from this a geometrical series of further dilutions down to $^1/_{100}$, for example, in the following ratios, in which each tube contain about ·8 as much of the gastric juice as the one that precedes it.

No.	1	2	3	4
Gastric juice diluted 50 times	2·0	1·6	1·3	1·0 c.c.
Water	0	·4	·7	1·0 c.c.

A control tube containing 2 c.c. of the unit rennin solution is placed in the middle of the series. To each tube an accurately measured volume of 2 c.c. of the calcium chloride-milk mixture is now added as rapidly as possible, the time is noted and the above-described procedure is followed. If the clotting of the control takes place between that of tubes 1 and 2, but nearer that of the former, an approximate interpolation gives 1·9 c.c. as the most probable value of the amount of gastric juice required. For very accurate work this could be confirmed by a series of yet more delicate tests between the limits 2·0 and 1·6 c.c.

The calculation of the relative concentration of rennin in the original gastric juice is as follows: 1·9 c.c. of the 1 in 50 dilution of gastric juice has the same activity as 2 c.c. of the control solution. So the diluted gastric juice contains $\frac{2}{1\cdot9}$ times as much enzyme as the control, and the original undiluted juice, therefore, $\frac{50\times2}{1\cdot9}$ times as much. Since we have already defined the control as containing one unit of ferment per c.c. the gastric juice contains $\frac{50\times2}{1\cdot9}=52\cdot6$ units per c.c.

The experimental error amounts to ±10 per cent of the total value, but can be reduced to ±5 per cent. by means of a third series of delicate tests as already mentioned.

It must be noted that it is necessary to keep all the tubes at the same temperature. All the solutions should be allowed to stand until they have acquired the temperature of the room, and

the tubes should be handled as little as possible. The use of a water bath may then be dispensed with.

Neutralisation of the gastric juice before the determination is not permissible as, under some circumstances, it may lead to the destruction of a part of the rennin, and so introduce errors. In any case it is not necessary, for the milk itself is a good buffer (v. infra).

EXERCISE 2.

Quantitative Estimation of Pepsin in Gastric Juice.[1]

Human blood serum diluted to 12 times its volume with distilled water or sheep's serum similarly diluted 15 times is treated with 10 per cent. sulpho-salicylic acid solution until a milky turbidity appears and the mixture just imparts a violet but not pure blue tint to Congo-red paper. If this is carried out correctly a homogeneous milky non-settling suspension of protein is obtained.

1 c.c. of the filtered gastric juice is taken in a test-tube, and in each of a series of five further tubes 1 c.c. of distilled water is placed. By means of a pipette 1 c.c. of the gastric juice is measured into the first of these five tubes, the contents are well mixed and 1 c.c. of this fluid is transferred to the next tube, and so on as described for the estimation of rennin. We thus have 1 c.c. of each of the following dilutions of gastric juice:

$^1/_1$ $^1/_2$ $^1/_4$ $^1/_8$ $^1/_{16}$ $^1/_{32}$

In a further tube is placed 1 c.c. of a unit pepsin solution made by diluting the original solution of Grübler's preparation, but in this case only 100 times. This same preparation can be used as a pepsin standard as well as a rennin standard, but it is convenient to use it 100 times stronger for the first purpose than for the second. 5 c.c. of the protein-salicylic acid mixture are now added to each of the tubes which are then all placed in a metal holder and immersed in a water bath at 38-40°C. As the action of the pepsin proceeds, the cloudy protein solutions gradually become clearer, and the object is to determine in which of the tubes this process is taking place at the same rate as in the control. For this purpose it is not necessary to wait for the clearing to become complete; it is possible to decide as soon as so much of the opalescence has disappeared that the control is readily distinguishable from the

[1] L. Michaelis and T. Rothstein l.c.

original material. As a rule from 5 to 25 minutes should be required for this; otherwise the control solution must be suitably diluted until this condition is fulfilled. When an approximate value has been obtained from this rough preliminary experiment, the determination is repeated with a more gradual series somewhat as follows. Starting with that dilution of the gastric juice which just shews a more rapid action than the control, the following further dilutions are prepared:

Diluted gastric juice c.c.	1·0	·8	·64	·5
Water c.c...	0	·2	·36	·5

A fifth tube contains 1 c.c. of the control ferment solution: to each add 5 c.c. of the protein-salicylic acid mixture and proceed as in the rougher experiment. The calculation is similar to that for the rennin estimation. If we take the unit of pepsin as the amount contained in 1 c.c. of the 100-fold dilution of the original rennin solution it follows that if the ratio of rennin to pepsin is the same for each specimen of gastric juice as for the Grübler preparation, we must always find 100 times as many units of rennin as of pepsin in any given case.

The accuracy of the pepsin estimation amounts to at least ± 20 per cent. of the total value.

The reason why a relatively small volume (1 c.c.) of the gastric juice is treated with such a large excess (5 c.c.) of the acid protein solution is as follows; it is necessary that in each experiment the contents of each tube, including the control, should possess the same acidity. This condition can be fulfilled only if it is ensured that the acidity is determined by the excess of the acid protein mixture which is added, so that the differences due to the various degrees of dilution of the gastric juice will be negligible.

II.

Threshold Concentrations for Precipitation from Colloidal Solution.

According to the historical definition a colloidal solution is one whose dissolved solid will not diffuse through a membrane of bladder or parchment when this is interposed between the solution itself and a portion of the pure solvent. This may be the case a priori under two conditions: either the molecules of the dissolved substance may be too large to pass through the pores of the membrane, or, while the single molecules may not be too large, the ultimate particles in the solution may not consist of single molecules but of aggregates (micellae), to permit the passage of which the pores may be too small. It is found as a matter of experience that large molecules often tend to be incompletely dispersed in this way, particularly when they possess no electric charge (i.e. form no ions). Colloidal solutions may be regarded as heterogeneous (micro-heterogeneous) systems consisting of a dispersion medium and a disperse phase (Wo. Ostwald).

Colloids may be classified according to several systems: for example into:

Spontaneous and non-spontaneous colloids, according as the colloid-former goes spontaneously into solution (albumin) or not (gold, mastic) when brought into contact with water.

Reversible and irreversible, according as to whether the condition of the colloidal solution is determined unequivocally and reversibly by its content of the colloid-former, of water and of other substances, particularly electrolytes, and by the temperature: in particular as to whether the colloidal material, after it has been precipitated by an electrolyte will re-dissolve and form the colloidal solution again when the precipitating agent is removed.

Hydrophil and hydrophobe, according as to the degree of affinity that is supposed to exist between the dispersed particles and the water.

Viscous and non-viscous, according as they appreciably increase the viscosity of water or not.

Emulsoids and suspensoids, according as the disperse phase is regarded as possessing the properties of a solid or of a liquid.

Colloids sensitive and colloids insensitive (i.e. less sensitive) to electrolytes.

The application of each of these criteria leads to the same general, if not completely identical, grouping of the colloids; and in each case some substances are found to occupy a position intermediate between the two extremes.

The mode of preparation of a spontaneous or reversible colloidal solution differs in no respect from that of an ordinary solution. The solid substance (albumin, for example) is brought into contact with water or the appropriate salt solution, and its distribution throughout the solution is accelerated by stirring. The solution of an irreversible colloid can be obtained only by indirect means which Wolfgang Ostwald has classified into dispersion and condensation methods respectively. In both cases it is a question of causing the colloid particles to be formed in a solvent under conditions that tend to maintain the stability of the suspension. One of the most important of such conditions is the electric charge on the particles relative to the solvent ; the greater the charge, other things being equal, the greater the stability of the colloidal solution. The magnitude of the charge depends, however, on the chemical nature of the substance concerned; colloid-formers with acid properties (mastic, fatty acids) usually acquire a strong negative charge in an alkaline solution; substances with basic properties, on the other hand, take on a powerful positive charge in acid solutions; while substances which are amphoteric (coagulated egg-white, alumina) will go into solution in both alkaline and acid media except only when they possess that particular approximately, but not necessarily exactly, neutral reaction at which the charge on the colloial particles is entirely suppressed. (*See* p. 63.)

Dispersion can be brought about by mechanical means such as grinding trituration, etc. (Indian ink, colloid mills), or shaking (oil in faintly alkaline water), or, in the case of metals by Bredig's method of disintegration in the electric arc. The easiest example of this is furnished by the preparation of colloidal silver, in which two silver wires of about 1 mm. diameter insulated almost to the ends by means of glass tubes are connected to the electric lighting mains and brought together under water so that an arc carrying a current of 5–10 amperes is struck between them. The best solvent to use is water rendered just alkaline with sodium carbonate.

All the condensation methods have this in common that the material that forms the starting point for the preparation of the colloid is at first in true molecular solution, either as a water-soluble compound (gold as gold-chloride) or in a solvent in which it forms a molecular dispersion (gum mastic in alcohol). Conditions are then established that lead to the precipitation of the actual colloid-former in an insoluble state (the gold chloride is reduced to gold by means of a reducing agent, the alcoholic gum solution is diluted with water) in a medium whose properties are such that the insoluble particles become highly charged with reference to the liquid. This involves the maintenance of a sufficiently small concentration of electrolytes, for, in general, electrolytes tend to suppress the charge on colloidal particles.

Examples of the condensation methods are afforded by the preparation of mastic sol (see exercise 4) by the dilution of the alcoholic solution with water and the preparation of gold sol by the reduction of gold chloride by such a reducing agent as formaldehyde (Zsigmondy[1]): 120 c.c. of doubly-distilled water (stored in Jena flasks) are brought to boiling in a 300-500 c.c. Jena beaker. During the heating 2·5 c.c. of a solution of auric chloride ($HAuCl_4$, $4H_20$, ·6 gm. in 100 c.c. of distilled water) and 3 to 3·5 c.c. of a ·18N solution of the purest K_2CO_3 are added. As soon as the liquid is boiling, 3 to 5 c.c. of formaldehyde solution (·3 c.c. of "formalin" in 100 c.c. of distilled water) are added in small portions at a time with constant stirring by means of a Jena glass rod. The red colour of the gold sol develops in one minute at the most.

An example of a different type is furnished by Graham's method for the preparation of ferric hydroxide sol. A 20 per cent. solution of $(NH_4)_2CO_3$ is added drop by drop to 100 c.c. of a half saturated solution of ferric chloride until the precipitate which is first formed is completely re-dissolved on shaking. The ferric hydroxide here goes spontaneously into a colloidal solution, from which the electrolytes are removed as completely as possible by dialysis.[2] But the ferric hydroxide as a result becomes an irreversible colloid, i.e. when it has been once more precipitated by the re-addition of electrolytes (see exercise 3) it will not re-dissolve when these electrolytes are dialysed away.

We will study first some irreversible colloids that are sensitive

[1] R. Zsigmondy, *Liebig's Ann*, **301**, 30, 1898.

[2] This experiment can be carried out in conjunction with exercise 41.

to electrolytes and determine the threshold concentrations required for their precipitation.

By far the most important factor in the precipitating action of a salt is the nature of that one of its ions that carries a charge of opposite sign to that carried by the disperse phase of the colloid (Hardy's rule): for this ion may even produce a complete reversal of the sign of the charge on the colloid. Polyvalent ions are more active in this respect than univalent (Schultze's rule) but among univalent ions $H^{\cdot}$ and OH' are distinguished by their particularly powerful effects.

Since the estimation of $H^{\cdot}$ and OH' ion concentration demands special methods we shall consider first the technically simpler study of the precipitating action of neutral salts, and only incidentally mention the action of solutions containing high concentrations of $H^{\cdot}$ or OH' ions, i.e. of strong acids or bases, reserving the special study of the actions of $H^{\cdot}$ and OH' ions for a later chapter.

The valency of an ion is by no means the only factor that determines its precipitating power; specific properties such as the degree of hydration of the ion and its discharge potential also play a part. The smaller the discharge potential the greater the precipitating power; thus it comes about that the noble metal silver is one of the most powerfully precipitating ions although it is only univalent. In the case of univalent ions the position in the periodic classification of the elements is of importance.

EXERCISE 3.

The precipitation of colloidal (electropositive) ferric hydroxide by electrolytes.

Ferric hydroxide is a positively charged hydrophobe colloid. It is precipitated irreversibly by all electrolytes. The nature of the cation is of practically no consequence, but the action of the anion is very closely dependent on its nature and particularly on its valency. Among anions, the OH' ion acts as a particularly powerful precipitant although it is only univalent.

A solution obtained by diluting the commercial "liquor ferri oxidati dialysati" 10-fold with distilled water may be used.

A series of 6–7 test tubes is taken and 9 c.c. of distilled water are measured into each except the first. Into this first tube 9 c.c. of molar KCl solution are placed; then 1 c.c. of the same solution

is measured into and mixed with the water in tube No. 2. 1 c.c. of this mixture is then transferred to the next tube, from which in after mixing a c.c. is transferred to the next and so on along the whole series. We thus obtain a quite rough geometrical series of KCl concentrations with the factor 10. To each tube is now added 1 c.c. of the 10-fold dilution of "dialysed iron." Precipitation takes place immediately in tube No. 1, after a time in No. 2 and not at all in No. 3 or any other.

If now we repeat the experiment, using instead of molar KCl M/2 $CaCl_2$ or M/3 $AlCl_3$, the threshold precipitating concentration is found to be practically the same, $AlCl_3$ having, however, a slightly weaker action than KCl of the same chlorine ion concentration.[1] If, however, M/2 Na_2SO_4 is tried immediate precipitation will be observed in all tubes up to No. 4.

Further it is easy to shew that the ferric hydroxide is readily precipated by even traces of NH_3 or NaOH, but not by hydrochloric or acetic acids.

EXERCISE 4.

The Precipitation of Electronegative Gum-Mastic by Electrolytes.

A filtered solution of 5 gms. of gum-mastic in 100 c.c. of 96 per cent. alcohol is prepared. 10 c.c. of this solution are placed in a large beaker and then 200 c.c. of distilled water are poured in in one portion as rapidly as possible. A milky fluid results, which should be filtered to remove any large flakes that may have been formed. With this liquid the same serial experiments are carried out as with the ferric hydroxide sol. If the tubes are allowed to stand for one hour before observation the threshold concentrations of the various salts required for the precipitation turn out to be as follows:

KCl	·1 normal.
$\frac{1}{2}$ $CaCl_2$	·01 normal
$\frac{1}{3}$ $AlCl_3$	·0001 to ·00001 normal
$\frac{1}{2}$ Na_2SO_4	·1 normal

[1] This action of $AlCl_3$ is explained by the circumstance that owing to hydrolysis its solution reacts acid. The exceptional behaviour is not produced to any appreciable extent, if at all, by the $Al^{\cdots}$ ions but by the change of acidity. Although we shall not deal with the effects of small changes of acidity until the 4th section, it is appropriate to point out here how difficult it is to arrange any experiment in which the effects of acidity can be disregarded.

The several chlorides are here not equally active, as in the experiments on ferric hydroxide, but shew a marked increase of precipitating power as the valency of the cation increases. On the other hand, Na_2SO_4 has no stronger action than NaCl because the difference between the two salts lies in their anions. The mastic is precipitated by acetic and hydrochloric acids, but not by NH_3—a behaviour again opposite to that of ferric hydroxide.

In the case of the very powerful precipitants we meet with the so-called irregular series: small concentrations of the salt produce precipitation, while moderate concentrations are without effect, but yet higher concentrations again cause precipitation. Beginning with ·5 molar, we prepare according to the method of exercise 1, a set of at least 20 concentrations of $AlCl_3$ in diminishing geometrical series with the factor 2. To 5 c.c. of each of these solutions 5 c.c. are added of mastic sol of one-third the concentration previously used. The result will vary slightly according to the exact properties of the mastic preparation, but in general there will be precipitation in the strongest solutions of $AlCl_3$ down to ·002 molar, say; no precipitation in the solutions of medium strength down to about ·0001 molar; precipitation again in the yet weaker solutions, and lastly no effect in the most dilute solutions of all, below ·00003 molar. The explanation of these effects is found in the reversal of the charge on the colloidal particles by the aluminium ions; in the region of intermediate concentrations where no precipitation takes place the mastic is positively charged, and so is not precipitated by the aluminium ions which have a charge of the same sign, and the chlorine ion will only produce precipitation in fairly high concentration because it is but feebly active in this respect.

EXERCISE 5.

Colour changes in Congo Rubin.[1]

In many coloured colloidal solutions a diminution in the fineness of the dispersion is marked by a change of tint. This colour change accompanies changes of dispersion too small to lead to visible precipitation, and thus furnishes conclusive proof that the visible separation of a colloid is simply the end result of a gradual

[1] Wolfgang Ostwald, *Kolloidchem. Beihefte*, **10**, 179, 1919.

increase in the degree of aggregation of the particles. Thus a red solution of colloidal gold takes on a blue colour in the initial stages of precipitation by electrolytes. The most convenient material for the study of these phenomena is Congo-rubin. It is used in solution in boiled distilled water at such dilutions that a layer of the thickness of a test-tube is quite transparent. After thorough cooling the solution is divided into 10 c.c. portions in a number of test-tubes.

The tubes are arranged in separate series of four, and the solutions in each series are treated with diminishing quantities, e.g. 1, ·5, ·25, and ·12 c.c., respectively, of one of the electrolyte solutions to be mentioned, and then all brought to the same volume by the addition of distilled water. The electrolyte solutions to be used in the several series are the following: N. KCl, N.NaCl, N.NH_4Cl, N.KCNS, N.KOH, dilute baryta; M/2 K_2SO_4; M/2 $(NH_4)_2SO_4$; M/100 (= N/50) $CaCl_2$, M/1000 (= N/333) $AlCl_3$, N/250 HCl. Each tube soon takes on a colour intermediate between pure red and pure blue, the exact tint being determined by the concentration of electrolyte present. If after some 30 to 60 minutes we observe which concentrations of the various salts have produced the same amount of change as for example, ·25 c.c. of N.KCl, we find that NaCl and NH_4Cl are about as potent as KCl, while the same effect has also been produced by ·25 c.c. of the N/50$CaCl_2$ or the same volume of the N/333 $AlCl_3$ or of the N/250 HCl. The chlorides thus possess very different activities which are determined by the nature of their respective cations. The weakest action is shown by K, Na, and NH_4, which all possess equal precipitating powers: the divalent ion $Ca^{\cdot\cdot}$ is 50 times as powerful as these (calculating in equivalent concentrations) and the trivalent $Al^{\cdot\cdot\cdot}$ is 333 times as strong. But the hydrogen ion is also almost as powerful as $Al^{\cdot\cdot\cdot}$ although only univalent (N/250 HCl produced the same effect as N/333 $AlCl_3$). This is an example of the general principle that valency is not the only factor which determines the activity of an ion. The behaviour of the electronegative Cong-rubin is thus exactly parallel to that of the likewise electronegative gum-mastic.

The degree of dispersion of Congo rubin is influenced very much by temperature changes. If a solution that has been turned blue by the addition of salts be warmed its red colour returns; but the blue colour reappears on cooling. Congo-rubin goes

spontaneously and reversibly into solution: gum mastic, on the other hand, will not disperse itself spontaneously in water, and changes of state produced in its sol are irreversible.

If the tubes are examined again after 24 hours, it will be seen that only the solutions containing a particular appropriate concentration of HCl are still homogeneous. In all the others a blue precipitate has separated out, leaving a red supernatant liquid of more or less intense colour, according to the salt concentration. In the tubes containing the greatest salt concentrations the red tint may be scarcely visible; but where any colour can be seen at all it is a pure rose-red with no admixture of violet or blue. Only the solutions containing a particular concentration of $AlCl_3$ (which can be determined by serial experiments) have a homogeneous violet appearance and are free from sediment. The explanation of this is as follows: Congo-rubin forms a true solution in water and this is red. In the presence of salts its solubility is diminished, and the portion which is salted out is blue. It is the combined effect of the blue suspended matter and the red solution which gives rise to the violet colour of the mixture. The blue particles slowly sediment, leaving the red solution . In the presence of an appropriate concentration of $Al^{\cdot\cdot\cdot}$ ions, however, the charge on the colloidal particles is not merely neutralised but reversed, so that they remain dispersed and the mixture has a uniform violet colour.

The special point in the behaviour of Congo-rubin is it is always precipitated in a blue form while in solution it is always red. Congo red, which otherwise is a closely related substance, differs in that it is precipitated sometimes in a red form (for example by NaCl) and sometimes in a blue modification (by HCl).

EXERCISE 6.

The Adjuvent Action of Different Ions.

If a mixture of active ions be allowed to act together on a colloid they are found to influence each other's activity in various ways. In most cases a summation of their individual effects is observed, but in particular instances even a mutually antagonistic influence exists. The summation effect is the more usual in the case of irreversible colloids. The following is an example: Two

serial experiments are carried out using a ·1 per cent. solution of Congo-rubin as a starting point, as under:

Tube No.	1	2	3	4	5	6
N/100 acetic acid	0·3	·6	1·2	2·4	4·8	9·6 c.c.
Water	11·7	11·4	10·8	9·6	7·2	2·4 c.c.
·1% Congo-rubin	·5	·5	·5	·5	·5	·5 c.c

No.	1	2	3	4	5	6
N/10,000 acetic acid	·3	·6	1·2	2·4	4·8	9·6 c.c.
Water	10·7	10·4	9·8	8·6	6·2	1·4 c.c.
N·KCl	1·0	1·0	1·0	1·0	1·0	1·0 c.c.
·1% Congo-rubin	·5	·5	·5	·5	·5	·5 c.c.

The resulting colour changes are the same in both experiments, and somewhat as follows:—

1	2	3	4	5	6
red	red	violet	blue	blue	blue

But each tube of the lower series contains only one hundredth of the concentration of acetic acid present in the corresponding tube of the upper series: since the effect is the same in both, the activity of the acid has been in part replaced by that of the neutral salt KCl.

EXERCISE 7.

The Antagonistic Action of Different Ions.

The other case—that in which the ions antagonise one another's activities—was first described by Jacques Loeb. He shewed that the poisonous effects produced by certain monovalent ions, e.g. K. or Na, on living cells could be prevented by the simultaneous presence of a small quantity of a divalent ion such as Ca or Zn, even although the divalent ion if acting alone would also be poisonous. Until recently only a few cases were known in which this antagonism of ions could be imitated in simple chemical systems outside the living cell. Neuschloss found the phenomenon to be exhibited by sols of lecithin. A case previously described by Sven Odén has recently been re-investigated by Freundlich and Scholz, and the following directions for the demonstration of the effect are based on the experimental details given by these authors.

The colloid which is used is the so-called Odén's modification of sulphur sol. There is another modification of this sol which is formed when an alcoholic solution of sulphur is diluted with water. This latter has the properties of an irreversible colloid like the mastic sol already described; it does not shew the phenomenon of ionic antagonism. On the other hand, Odén's sol has the properties of a reversible colloid; on removal of the precepitating agent it goes spontaneously into solution again, and so shews a close resemblance to the reversible colloids of living cells with which the antagonism was first discovered.

Odén's sol is prepared as follows:—

An approximately molar (2N) solution of sulphurous acid is prepared by titration, using phenolphthalein as indicator. Through a 100 c.c. portion of this solution sulphuretted hydrogen is passed, when a yellowish milky solution of colloidal sulphur is at once produced. The passage of the gas is continued for about an hour until the smell of sulphur dioxide has practically disappeared from the liquid, which is then allowed to stand for 24 hours in order to allow the coarser particles to settle. The colloidal solution is then poured off: it is to be diluted 100 times with distilled water immediately before use. The following serial experiments are set up:

I. Tube No.	1	2	3	4	5	6
10 M.LiCl c.c. ..	·24	·32	·42	·56	·75	1·00
Water c.c. ..	·76	·68	·58	·44	·25	0
Sol c.c.	10·0	10·0	10·0	10·0	10·0	10·0

It will be found that tubes 4—6 shew immediately a thick opalescence while Nos. 1—3 remain quite clear. It is remarkable how sharply the threshold for the precipitation of this colloid may be defined. With different preparations it may not occur at exactly the concentrations employed in tube No. 4, but it is always within the limits of the series we have used.

II. Tube No.	1	2	3	4	5	6
·1 M. $MgCl_2$ c.c.	·10	·12	·14	·17	·20	·24
Water c.c. ..	·90	·88	·86	·83	·80	·76
Sol c.c.	10·0	10·0	10·0	10·0	10·0	10·0

This experiment gives us the threshold for magnesium chloride.

We now prepare a sol containing one quarter of the threshold precipitating concentration of LiCl, i.e. if in the above series tube No. 4 was the first to shew precipitation, 1·4 c.c. of the 10 M. LiCl solution are added to 100 c.c. of the diluted sol. This mixture is referred to in the following table as "Li Sol."

The following experiment is then carried out:

III. Tube No.	1	2	3	4	5	6
·1 M. $MgCl_2$ c.c.	·40	·48	·58	·70	·84	1·0
Water c.c. ..	·60	·52	·42	·30	·16	0
Li Sol c.c. ..	10·0	10·0	10·0	10·0	10·0	10·0

The precipitation threshold for magnesium chloride is now at ·84 c.c., while in the absence of the LiCl it was at ·17 c.c. In other words the LiCl has increased the threshold for $MgCl_2$ five times; The Mg. and Li together do not produce an additive effect, but antagonise each other's action.

EXERCISE 8.

The Mutual Protective Action and Mutual Precipitation of Colloids.

Colloids that are present together in the same solution reciprocally influence each other's sensitiveness to electrolytes. An insensitive colloid protects a sensitive of the same sign of charge against the action of electrolytes. On the other hand, a colloid of one sign will in certain proportions precipitate one of opposite sign or at least render it more sensitive to electrolytes.

(I.) A mixture is made of 10 c.c. of mastic sol (prepared as directed on page 12) and 1 c.c. of 1 per cent. gelatin solution. For comparison a second 10 c.c. of the mastic is diluted with 1 c.c. of distilled water. To each of these solutions, 10 c.c. of N. KCl are added, when in the tube containing no gelatin a coarse precipitate is rapidly formed, while in the tube with gelatin no effect is observed.

The various relatively insensitive colloids shew very different degrees of protective action when tested with a given sensitive one. The quantity of an insensitive colloid which will prevent the change of colour of a gold sol from red to blue under the influence of a fixed quantity of sodium chloride has been termed by Zsigmondy its "gold number." Wo. Ostwald measures the protective effect in terms of Congo-rubin and so uses the "rubin number." Into

each of a series of tubes is placed 1 c.c. of a 1 per cent. solution of Congo-rubin and varying quantities of the protective colloid. The mixtures are then all diluted to a uniform volume of 9 c.c. and lastly 1 c.c. of ·5N. KCl is added to each. The object is to determine the minimum concentration of protective colloid which will just prevent the appearance after 10 minutes of an appreciable change of colour from that of a control solution made up to the same volume but without KCl.

Such experiments can be arranged as follows:—

·1% Congo-rubin c.c.	1	1	1	1	1*	1*
1% gelatin c.c. ..	4	2	1	–	–	–
·5% gelatin c.c. ..	–	–	–	1	·5	·25
Water c.c.	4	6	7	7	7·5	7·75
·5 M.KCl c.c.	1	1	1	1	1	1

or:—

·1% Congo-rubin c.c. ..	1	1	1	1*	1*	
·1% hæmoglobin[1] c.c. ..	4	2	1	·5	·25	
Water c.c.	4	6	7	7·5	7·75	
·5 M.KCl c.c.	1	1	1	1	1	

In the experiments marked with an asterisk the protective action is not observed. The composition of the contents of the tubes immediately preceding these gives a measure of the protective power of the particular colloid—the concentrations of gelatin and of hæmoglobin in these tubes being the "rubin numbers" of these substances as already defined.

In these cases the protective colloid (for example, the gelatin in neutral solution) has invariably the same sign of charge as the suspensoid colloid (i.e. negative). If, however, the colloids are oppositely charged, in certain proportions they sensitise each other towards electrolytes, and under some conditions spontaneous mutual precipitation may occur. If the precipitate be examined it is found that not only has the more sensitive colloid been thrown down but that also more or less of the insensitive has been carried down as well. The following method for the removal of proteins from bloodserum depends on these facts.[2]

[1] Merck's "Soluble Hæmoglobin."

[2] P. Rona and L. Michælis, *Biochem. Zeitschr.* **7**, 329, 1908, and **16**, 60, 1909.

5 c.c. of blood serum are diluted with 50 c.c. of distilled water; 25 c.c. of five times diluted ferric hydroxide sol (Liq. ferri oxydati dialysati, not the Liq. ferri oxychlorati of the Phamacopæa) are then run in drop by drop with constant shaking. A precipitate is formed at once and can be readily filtered off after a few minutes. The filtrate is crystal clear and free from proteins. The ferric hydroxide is positively, and the serum proteins, in the approximately neutral solution, negatively charged.

The removal of the proteins from whole blood is a more difficult problem, partly because the hæmoglobin carries only a feeble negative charge, for its iso-electric point is not far removed from the approximately neutral reaction which obtains in the mixture. Ferric hydroxide does not produce spontaneous complete precipitation: the addition of electrolytes is also necessary. And on account of the protective action of the hæmoglobin a greater concentration of electrolytes is required than in a pure iron solution.

The method for the removal of proteins from blood is carried out as follows: 5 c.c. of defibrinated blood are diluted with 45 c.c. of water, and then 100 c.c. of the four-times diluted ferric hydroxide solution are run in very slowly and with constant stirring. Only incomplete precipitation takes place. After 10 minutes ·1gm. of finely powdered K_2SO_4 is added and the whole is well shaken. The precipitation now takes place rapidly, and after 5 minutes the mixture should filter, giving a clear filtrate, free from proteins and hæmoglobin. If, when large quantities of blood are being dealt with, a trace of hæmoglobin comes through in the filtrate this can be removed subsequently by the addition of a further small quantity of the iron solution.

This method of removal of proteins becomes very certain when combined with heat-coagulation. We will describe the form in which it is adapted for use in the micro-estimation of the blood sugar.[1]

1 c.c. of blood (rendered incoagulable with NaF, or if only for practice in the method, defibrinated) is diluted in a 100 c.c. flask with 2 c.c. of distilled water, of which a certain amount may be used for washing out the pipette. The solution is heated to boiling, kept boiling for two seconds and then removed from the flame. Then 7·5 c.c. of the five-times diluted ferric hydroxide solution

[1] L. Michaelis, *Biochem. Zeitschr*, **59**, 166, 1914.

are added drop by drop with continuous shaking, and finally ·5 c.c. of ·5 per cent. $MgSO_4$ solution. The mixture can be filtered at once. The filtrate is clear and colourless, and comes through no more slowly than pure water; it is free from proteins. Rather more than half of the total volume of the mixture can be obtained as filtrate, but not the whole, so that from the estimated quantity of sugar in a known aliquot part the total sugar present in the whole must be calculated.

EXERCISE 9.

Hofmeister Series in the Precipitation of Proteins by Ions.

Even hydrophil colloids such as proteins can be salted out by a great variety of substances, although only when these are present in high concentrations. This salting out effect depends on the nature of both the anion and the cation of the salt used. It depends also on the sign of the charge which is carried by the protein—and this may vary. For the sake of clearness we will deal first with a protein in distinctly acid solution in which it is definitely positively charged.

5 c.c. of blood serum are diluted with 50 c.c. of N/50 HCl. 2 c.c. of this solution are placed in each of a number of test-tubes. We then determine by trial how many c.c. of any given salt solution must be added to a tube in order to produce a distinct opalescence. If we use normal solutions of the under-mentioned salts we obtain the following results:—

(1) KCl—no precipitation at all, even after the addition of 12 c.c.
(2) KBr—well-marked opalescence with ·75 c.c.
(3) KI—well-marked opalescence with ·5 c.c.
(4) KCNS—well-marked opalescence with ·2 c.c.

(We do not trouble in this case to dilute all the solutions to the same volume.)

Thus if we change the anion only we find that their precipitating powers increase in the order: Cl, Br, I, SCN. This is the Hofmeister series of anions which is of very frequent occurrence even outside the domain of colloid chemistry. For example, the influence of these salts on the solubility of amino-benzoic acid is exactly analogous to the phenomenon we have just described.[1]

[1] Lundén, *Svensk. Vet. Akad. Arkiv. f. Kemi* 2, 11. 1905; see also H. Euler, *Chemie der Enzyme*, 2nd edn., 1920, p. 61. Here the effects of the same series of ions on the solubility of amino-benzoic acid is described.

EXERCISE 10.

The Hofmeister Series with Hæmoglobin.[1]

A very suitable material for the study of ionic series is furnished by the commercial so-called "hæmoglobin" because it is soluble in neutral, acid and alkaline media (as is albumin, but not casein or globulin) and because it is precipitated by even moderate concentrations of relatively inactive salts (in contrast to albumin and gelatin). A 2 per cent. solution of Merck's "completely soluble, powdered" hæmoglobin is made by rubbing up the powder with a little water in a mortar and then gradually adding the remainder of the water and finally filtering.

The following salt solutions are then prepared, each having a total volume of 8 c.c., and to each 2 c.c. of the hæmoglobin solution are added:—

1. 2N. K_3-citrate c.c.	2	4*		1 Mol. $(NH_4)_2SO_4$	·5	1*	2	
Water c.c.	6	4		Water	7·5	7	6	
2. ·5 Mol. K_2SO_4	4	8*		1 Mol. Na_2SO_4	·5	1*	2	
Water	4	0		Water	7·5	7	6	
3. 2 Mol. K-acetate	2	4*		Li_2SO_4 satd. soln. (=2·3 Mol.)	8*	4		
Water	6	4		Water	0	4		
4. KCl satd. soln. (=3·5 Mol.)	4	6*	8	$CaCl_2$ or $MgCl_2$ ·01 Mol.	8	4*	2	
Water	4	2	0	Water	0	4	6	
5. KNO_3 satd. soln. (=2·4 Mol.)	8*			$AlCl_3$ satd. soln. (=about 9 Mol.)	5*	2·5	1·25	
Water	0			Water	3	5·5	6·75	
6. KCNS satd. soln. (=about 14 Mol.)	8	(usually no precipitation)[2]						
Water	0							

[1] Quoted from Wo. Ostwald, *Kleines Praktikum der Kolloidchemie*. Dresden and Leipzig, Theod. Steinkopf, 1920.

[2] Recent preparations of "hæmoglobin" have been most powerfully precipitated by KCNS; apparently they are somewhat acid. The uncertain reproducibility of this experiment is a striking example of our warning that no certain statement as to the precipitating power of a salt can be made without an exact knowledge of the acidity or alkalinity of the solution. See the preliminary experiments on the subject on p. 23 and the more detailed account in section IV.

In each case the solution in which immediate precipitation occurs is denoted by means of an asterisk.

On comparing the various potassium salts among themselves it is seen that the anions arrange themselves as follows in order of increasing precipitation power:

$$CNS', \quad NO'_3, \quad Cl', \text{ acetate}, \quad SO''_4, \text{ citrate.}$$

On the other hand, comparing all the chlorides or sulphates, the cations are seen to increase in precipitating power in the order:

$$Li, \quad Na, \quad K, = NH_4.$$

This applies to hæmoglobin in neutral solution.

In alkaline solution the relationships are as follows:

2 c.c. of the hæmoglobin solution are immediately precipitated when added to a mixture of 2 c.c. of saturated (4 Mol.) $(NH_4)_2SO_4$ solution + 6 c.c. water + 6 drops of 1 N. NaOH.

No precipitation occurs when 2 c.c. of the hæmoglobin solution are added to a mixture of 8 c.c. of saturated (8 Mol.) NH_4CNS solution + 6 drops of 1 N. NaOH.

The order of the anions CNS'—SO_4'' is thus the same as before.

In acid solution:

2 c.c. of the hæmoglobin solution are immediately precipitated when added to a mixture of 2 c.c. of 1 Mol. $(NH_4)_2SO_4$ + 6 c.c. water + 6 drops of N. HCl.

Immediate precipitation also occurs when 2 c.c. of hæmoglobin solution are added to a mixture of 2 c.c. of ·2 Mol NH_4CNS + 6 c.c. water + 6 drops of N. HCl.

The order of the anions is now reversed: CNS' precipitates more powerfully than SO_4'' in acid solution.

On the other hand the order of the univalent cations is not reversed by this degree of acidity; although the absolute values of their threshold concentrations are changed, for example:

Alkaline	Li	1 Mol.	NH_4	·8 Mol.	K ·5 Mol.
Acid	Li	·13 Mol.	NH_4	·08 Mol.	K ·025 Mol.

III.

A few Experiments on Optical Heterogeneity.

EXERCISE 11.

Thorough-going experiments on the optical resolution of colloidal solutions can be made only by means of Siedentopf and Zsigmondy's ultramicroscope fitted with a suitable trough; but such an instrument is not to be found in all laboratories and for many purposes the ultra-condenser is an efficient substitute. Every student of bacteriology becomes familiar with the phenomenon of Brownian movement in protein particles when using an ultra-condensor for the examination of diluted serum or exudates for spirochæts.

Some of the fundamental phenomena due to optical heterogeneity can be studied without the aid of the microscope if there is available a powerful source of light such as the arc lamp that is ordinarily used for the ultra-condensor.

By means of a bull's-eye condensing lens a cone of light is allowed to pass through a test-tube of very dilute mastic sol (prepared as directed on p. 12). The path of the rays is seen to shine out very brightly—the Tindall phenomenon. If this bright cone be now observed through a Nicol prism it will be found that its brightness varies according to the position of the prism, becoming alternately bright and dark as the prism is rotated through each 90°. In other words the light which is scattered by the colloidal particles is largely, if not completely, plane polarised.

The path of the rays is also visible in dilute solutions of fluorescent substances such as eosin, fluorescein and quinine as a bright band of the characteristic fluorescent colour of the material. But in this case the light emitted is not polarised, for it does not change in intensity on rotating a Nicol through which it is observed. The light emitted from a fluorescent substance arises from the individual molecules, and is not polarised; that which is given out from a colloidal solution is scattered by its relatively large suspended particles and is polarised.

In the case of coloured colloids the light which is scattered is often different in colour from that which emerges from the solution, the two being not uncommonly complementary. But with the

help of a Nicol prism it is always possible to decide in any given case whether true fluorescence or merely a scattering of certain coloured rays " pseudo-fluorescence " is occurring.

Pseudo-fluorescense can be readily observed as follows: a fragment of indophenol is dissolved in alcohol and the solution is then diluted several times with distilled water. The mixture still retains the blue colour of the original alcoholic solution when examined by transmitted light: but indophenol is not truly soluble in water; like mastic, it forms a sol, which, when illuminated from the side with the arc lamp, shews a red-brown cone of light which can be shewn to be polarised.

An extremely dilute colloidal solution of Prussian blue retains this colour by transmitted light, but when illuminated from the side shews a red cone which is also produced by pseudo-fluorescence.

A similarly dilute solution of Nile blue shews phenomena which are superficially identical with these; but in this case we are dealing with true fluorescence—the light is not polarised. But if the Nile blue solution is treated with NaOH its colour to ordinary observation changes to the red tint of the fluorescence of the original untreated solution. The cone of light produced by transverse illumination has, in this case, the same colour, but it is polarised, which means to say that the Nile blue is no longer in true solution, but has formed a colloidal solution as a preliminary stage in its complete precipitation.

Changes in degree of dispersion are very commonly associated with changes of colour, but not always in the same direction. For example, sols of gold or Congo-rubin change from red to blue, but those of Nile blue, toluidine blue, etc., from blue to red, as the size of the colloidal particles increases.

IV.

The Estimation of Hydrogen Ions by means of Indicators.

(α) The special relationships of H· and OH′ ions.

It is impossible to obtain a clear insight into the effects of any ions without at the same time taking into account those of the H· and OH′ ions. For in dealing with an aqueous solution of any electrolyte we are never concerned with its own particular pair of ions alone, but always also with the ions H· and OH′ which are never completely absent from any aqueous fluid. Now since these are among the most active of ions, their effects must be borne very clearly in mind, even in neutral solutions in which their total concentration is a minimum. All the results we have obtained in the preceding section for ionic precipitation thresholds must be regarded as entirely preliminary, and will take on a quite different aspect after exercises 23 to 25, for example, have been studied. One small example of the importance of these considerations: we estimated the threshold concentration of NaCl required for the precipitation of mastic sol in "neutral solution." But what we were really measuring in this case was the concentration of NaCl that just overcame the dispersing action of the OH′ ions, and whose aggregating effect, added to that of the H· ions, was sufficient to produce visible precipitation. There is no absolute threshold concentration of NaCl but only a relative threshold which must be referred to the OH′ ion concentration of the solution and whose value changes with the smallest alteration in that OH′ ion concentration. And seeing that our mastic preparations will, in general, depart a little from exact neutrality the threshold value we have found for NaCl will have no particular fundamental significance unless we can say to what OH′ ion concentration it applies.

Or to take another example: J. Traube found that all surface active substances have a powerful pharmacological action. Now the alkaloids shew a conditioned surface-activity, i.e. the surface tension of their solutions is affected by the ions which are also present in the liquid, and in particular, again, by the concentration of

OH′ ions. If now it is a question of the determination of the threshold value for the effect of quinine for example on an enzyme action or for its bactericidal action on a bacterial emulsion it must be realised that this threshold can never possess an absolute value, but must always be stated with reference to the prevailing H·-ion concentration on which its value depends. Such examples could be multiplied indefinitely.

But for the estimation and standardisation of hydrogen ions special methods are required. If we wish to produce a given concentration of Cl′ ions in a solution we simply add the appropriate quantity of NaCl: since this salt is practically completely ionised the chlorine ion concentration in the solution will be nearly equal to that of the NaCl. But if we add some acetic acid to a solution we cannot predict from the quantity of acid added the H·-ion concentration that will be produced; for acetic acid is ionised to only a small and variable extent which depends on the previous composition of the solution, and so on. Hence the need for the special methods of standardisation and estimation to which we have referred. For the preparation of solutions of standard H·-ion concentration "buffer" substances are used; for the estimation of H·-ion concentrations there are two chief methods in use—the electrometric and the indicator method; of these we shall study the latter first.

The special nature of the H· and OH′ ions is shewn further by the fact that numbers of acids and bases are but feebly dissociated in aqueous solution, while practically all other electrolytes are very strongly ionised. This only applies, however, to aqueous solutions or, speaking generally, to those whose solvents possess a high dielectric constant. In organic solvents of low dielectric constant ("oils") salts, acids and bases all seem to be but feebly dissociated and the difference between strong and weak acids, so important in aqueous solutions, seems to disappear. These relationships await further investigation, but important beginnings have already been made for which see "Die Entstehung elektrischer Ströme in lebenden Geweben" by R. Beutner, Stuttgart, 1920. The importance of such a study will be evident when on the one hand it is remembered how important a rôle "lipoid" phases play in living tissues and when, on the other hand it is realised that all the methods and results which we shall study in the following exercises are applicable only to aqueous solutions.

(*b*) Units and Nomenclature.

The concentration of H·-ions is expressed in gm.-ions per litre: it is usually represented by the symbols C_{H^+}, [H] or [H·]. In the following pages we shall denote it simply by "h" and refer to it as the "hydrogen-ion number."

The meaning of the symbol pH "the hydrogen-ion exponent" is as follows:[1]

$$pH = -\log_{10} h = \log \frac{1}{h}$$

We shall write the concentration of OH'ions as oh (the "hydroxyl-ion number") and define the corresponding "hydroxyl-ion exponent" as

$$pOH = -\log_{10} oh = \log \frac{1}{oh}$$

The product h·oh = kw, the ionisation constant of water. In every aqueous solution, whether acid, neutral or alkaline—

$$h \cdot oh = k_w$$

or $$pH + pOH = p_{kw}.$$

where p_{kw}, according to the above nomenclature, stands for $-\log. k_w$.

k_w = at 18°C.	$\cdot 72 \times 10^{-14}$	at 37°C.	$3 \cdot 2 \times 10^{-14}$
p_{kw} = at 18°C.	14·14	at 37°C.	13·50

At the neutral point the following relations hold:

at 18°C. $h = oh = \cdot 85 \times 10^{-7}$; $pH = pOH = 7 \cdot 07$

at 37°C. $h = oh = 1 \cdot 77 \times 10^{-7}$; $pH = pOH = 6 \cdot 76$.

At acid reactions h is $>$ at the neutral point

pH $<$,, ,, ,,

oh $<$,, ,, ,,

pOH $>$,, ,, ,,

The commoner indicators change colour at the following reactions:

Methyl Orange	at about	pH 4
Methyl red	,, ,,	pH 6
Litmus	,, ,,	pH 7
Phenolphthalein	,, ,,	pH 8

[1] S. P. L. Sörensen, *Biochem. Zeitschr*, **21**, 131, 1909.

pH of the circulating blood is = 7·35 to 7·40
of urine . . . . . 5 to 7
of nutritive broth . . . . 7 to 7·5
of ordinary distilled water, not freed from CO_2 . . . . . towards 6 or even 5
of fresh tap water . . . 7·5 to 7·6
of gastric juice . . . . . 1·5 to 2

Examples of the intercover ion of h and pH:

(1) Let $h = 2{\cdot}0 \times 10^{-5}$,
then $\log. h = \log. 2 + \log. 10^{-5}$
$= {\cdot}30 - 5 = -4{\cdot}70$
$pH = +4{\cdot}70$

(2) Let $pH = 6{\cdot}70$
then $\log. h = -6.70 = \dot{+}{\cdot}30 - 7$
$h = 2{\cdot}0 \times 10^{-7}$

A geometrical series of values of h in multiples of 2, e.g.

$1 \cdot 10^{-5}$ $2 \cdot 10^{-5}$ $4 \cdot 10^{-5}$ $8 \cdot 10^{-5}$

corresponds to an arithmetical pH series with a constant difference of ·3, thus:

5·0 4·7 4·4 4·1

Thus a series which satisfies the condition of a geometrical increase of h is an arithmetical series of pH values.

In order to facilitate the conversion of h values into pH and vice-versa a table of three figure logarithms is appended at the end of the book. The first figure of the number is to be found in the left-hand column, while the second (or second and third, as the case may be) are to be read off in the topmost horizontal line. Below each mantissa is entered in italics the difference between itself and 1000: this is of use in the calculation of pH values.

This table gives one more figure in the mantissa than is necessary for the calculation of pH in view of the possible experimental error of the measurement. The pH values should always be rounded off to two decimal places.

The number of places given in the table is generally sufficient for most of the problems that arise in the laboratory. It can be used for general calculations, and by its means not only multiplication but also division can be performed by the addition of mantissae, the upper figures being used in the former case and the lower figures (in italics) in the latter.

EXERCISE 12.

Buffer Solutions.

(a) In a solution containing both a weak acid and one of its salts h is practically entirely dependent on the ratio of acid to salt and is hardly affected at all by the actual quantities of these substances present. So that dilution with water will produce very little change in h. This seeming paradox can be explained in an elementary fashion as follows: A weak acid is only feebly ionised and its degree of ionisation will be still further depressed in the presence of its own salt. If the solution be diluted the concentration of the acid certainly is reduced, but so also is the concentration of the salt, and with it to a corresponding extent the depression of the ionisation of the acid.

A N. solution of crystallised sodium acetate ($CH_3COONa+3H_2O$) is made up by dissolving 13·61 gms. of the salt in 100 c.c. of distilled water. N. acetic acid is also prepared by titration against N. NaOH using phenolphthalein as indicator. From these N/10 solutions are made by dilution. For the demonstration of the buffer effect the following mixtures are prepared:

No.	1	2	3	4
·1 N. sodium acetate c.c.	5	7	8·5	9
·1 N. acetic acid c.c.	5	3	1·5	1

These quantities are not chosen to correspond with the terms of any particular series, but so as to give suitable colour changes with the indicator used.

A second series of four tubes is next taken, each containing 9 c.c. of water, and 1 c.c. of the mixture in each tube of the first series is transferred to the corresponding tube of this second series. One or two drops of methyl red solution (·1 gm. in 300 c.c. 90 per cent. alcohol+200 c.c. water) are then added to each of the 8 tubes, the same quantity to each. The first four tubes now shew a series of tints ranging from pure red to pure yellow. And each tube of the second series shews the same tint as that of the corresponding tube of the first series although the buffer solution it contains has been diluted ten times. h thus depends on the ratio of the molecular concentrations of free acetic acid to sodium acetate. As an approximation we may write:

$$h = k \cdot \frac{\text{(free acid)}}{\text{(Na-salt of the acid)}} \qquad (1)$$

where k is the dissociation constant of the particular acid. In round numbers the values of k for some common acids are as follows:

Tartaric acid	1×10^{-3}
Lactic acid	$1{\cdot}5 \times 10^{-4}$
Acetic acid	2×10^{-5}
Sodium di-hydrogen phosphate ..	2×10^{-7}
Carbonic acid	3×10^{-7}

Di-hydrogen sodium phosphate (primary sodium phosphate) is here regarded as an acid; its sodium salt is di-sodium hydrogen phosphate (secondary sodium phosphate). The above rule does not apply to strong acids such as HCl. The buffering of tissue fluids is as a rule brought about by a mixture of H_2CO_3 and $NaHCO_3$. Na_2CO_3 does not occur in living tissues.

If a solution contains two buffers, such, for example, as (1) $H_2CO_3 + NaHCO_3$ and (2) $NaH_2PO_4 + Na_2HPO_4$ its value of h can be calculated from either the ratio of H_2CO_3 to $NaHCO_3$ or the ratio of NaH_2PO_4, to Na_2HPO_4. For if, for example, H_2CO_3 and Na_2HPO_4 are mixed these substances react in such a way that h has the same value whether calculated from the bicarbonate or from the phosphate concentrations.

In blood the bicarbonate system is the chief buffer, in urine the phosphate system.

The above formula (1) for h is only approximate. Usually h is found to be a little larger than the formula would lead one to expect. For greater accuracy we should write:

$$h = k' \cdot \frac{(\text{free acid})}{(\text{Na-salt of the acid})}$$

where k′ is a little greater than the dissociation constant of the acid as determined by conductivity measurements. The value of k′ is dependent on the total salt concentration of the solution; for a mixture containing only small quantities of electrolytes it approximates in value to the true dissociation constant k; a salt concentration of ·I N. will increase its value by about 10 to 15 per cent. and a concentration of 1 N. by about 25 per cent. (in the case of carbonic acid mixtures 100 per cent.).

Until recently this was explained as follows: The factor which determines the degree of depression of the ionisation of the acid is

the concentration of acid anions formed by the dissociation of the sodium salt. Now in general the salt was supposed not to be completely ionised so that the term Na-salt concentration in the denominator of the fraction should be multiplied by the degree of ionisation δ which will vary with the concentration and, in any case, will be always less than 1. But more recently the view has been put forward[1] that the sodium salts are always practically completely dissociated, but the active masses of their ions (in the sense of the law of mass action) are reduced by their mutual electrostatic attraction.

(b) A mixture is made of 10 c.c. N. acetic acid + 1 c.c. of the N. sodium acetate (pH = about 3·7) and to this is added one drop of a solution of methyl orange made up as directed for methyl red. The solution is coloured orange. In a second tube a drop of the indicator is added to 10 c.c. of physiological NaCl solution and then ·0IN. HCl is added drop by drop until the tint of the mixture is as nearly as may be the same as that of the acetate solution. If now to each tube ·5–·6 c.c. of a 1 per cent. gelatin solution be added the colour of the acetate solution remains unchanged, but that of the HCl mixture changes to pure yellow. The h of the acetate buffer is thus hardly affected by the addition of a substance (gelatin) which is capable of combining with acids, while that of the unbuffered HCl solution is very considerably reduced.

The two acid solutions, originally of the same reaction, possess, as one may say, the same acid intensity but very different acid capacities. The different acid capacities or "buffer values" of two solutions of the same h may be further illustrated by the following experiment:

(c) A mixture is made of 10 c.c. of N, acetic acid + 1 c.c. of N. sodium acetate. 1 c.c. of this mixture is transferred to a second vessel and there diluted with 9 c.c. of distilled water. Methyl orange is added to both solutions; in accordance with the principles developed above the tint is the same in both. If now both solutions are contaminated with some substance capable of neutralising acid for example by adding ·1 N. NaOH drop by drop it will be seen from the colour change of the indicator that the reaction changes much more readily in the diluted than in the undiluted buffer.

[1] N. Bjerrum, *Z. f. Elektrochemie* **24**, 321, 1918.

From these experiments we establish the following conclusions with regard to buffer action: A mixture of a weak acid with its alkali salt has the properties of a buffer, namely:

1. Its h is hardly changed on dilution with water.

2. Its h is less changed by the addition of substances capable of combining with acids than that of an unbuffered solution of the same original reaction.

3. By dilution with water, although its h is not appreciably changed, the stability of its h against contaminating substances (its " buffer value ") is diminished.

If, therefore, we are faced with the problem of producing a given h in any solution we add to it a buffer which possesses this particular value of h, and the problem is at all events approximately solved. In using this method for the establishment of a definite value of h it is always necessary to control the process by a subsequent confirmatory measurement. For the method can only be approximate because the acid capacity of a buffer is in any case limited.

EXERCISE 13.

Sörensen's Indicator method of Estimation of Hydrogen-Ion Concentrations using Standard Buffer Solutions.

The principle of the method is as follows: A series of stock solutions is made up from which by mixture in appropriate proportions " buffer solutions " of definite h can at any time be prepared. If now it is a question of the determination of h in any given solution a suitable indicator is added and then various mixtures of the stock solutions are investigated until a buffer solution is found that gives the same tint with the indicator as the unknown fluid. The values of h for the various buffer mixtures have been determined once for all by means of the electro-metric method (see chap. XII.): the h of the unknown liquid is equal to that of the particular buffer mixture that gives the same colour with the indicator.

For the determination of the h of fresh tap water, which shall form our first exercise in the method, only two stock solutions are necessary:

(1) An M/15 solution of primary potassium phosphate.

9·078 gms. of the salt are dissolved in distilled water and diluted

[1] S. P. L. Sörensen, *Biochem. Zeitschr*, **21**, 131, 1909.

to 1 litre. In order to expel carbon dioxide the water used for this solution must be heated to boiling in a vessel of tinned copper or Jena glass, kept boiling for 5 minutes, and then cooled out of contact with the CO_2 of the atmosphere. This latter condition is ensured by fitting the flask with a bored rubber stopper carrying a soda-lime tube. The salt is first dissolved in a little of the water, while it is still fairly warm, in a measuring flask, then after thorough cooling the solution is diluted to 1 litre and at once transferred to a Woulf's bottle of which one neck is connected with a protecting soda-lime tower and the other is fitted with a burette with automatic zero setting. (Fig. 1). The upper end of the burette is also provided with a soda-lime tube, which is kept closed by a stopper except when the apparatus is in use. Similarly the soda-lime tower is also kept stoppered except when the solution is required, when the rubber blow-ball is attached.

2. An M/15 solution of Sörensen's secondary sodium phosphate. 11·876 gms. are dissolved and made up to 1 litre with the same precautions and stored in the same manner.

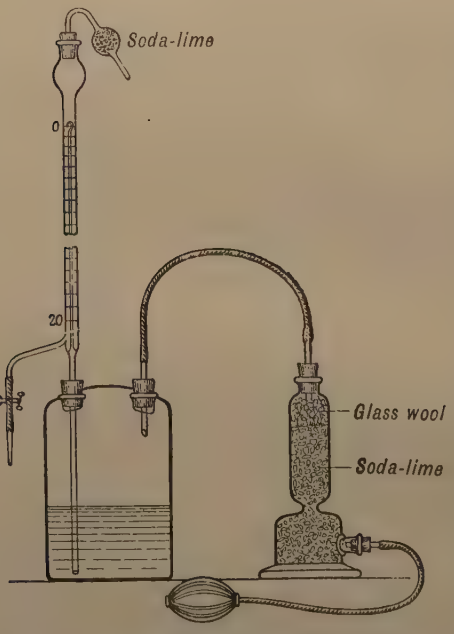

FIG. 1.—Storage bottle with burette for stock buffer solutions.

This Sörensen's secondary sodium phosphate differs from the ordinary secondary sodium phosphate in its content of water of crystallisation. The ordinary phosphate contains six molecules of water of crystalisation, but readily effloresces so that its composition is not definite. Sörensen's salt contains only 2 H_2O and is stable. It is obtained from the ordinary phosphate by allowing it to effloresce in the air by exposing it for several weeks in the powdered condition in shallow dishes. But it is better to ensure the completeness of the efflorescence by warming, so that the following procedure is recommended: about 1·5 times the required amount is taken from the stock of the well effloresced Sörensen's salt and kept in a thin layer in an incubator at 36°—38°C. for one or two days. It is then allowed to cool in the dessicator before weighing out. The salt so obtained has a constant water content of exactly $2H_2O$.

3. Indicators.

Methyl red (Palitzsch) ·1 gm. dissolved in 300 c.c. of about 93 per cent alcohol + 200 c.c. distilled water.

p-Nitrophenol, ·4 g.m. in 60 c.c. alcohol + 90 c.c. water.

Neutral red, ·01 per cent. solution in 50 per cent. alcohol.

α–Naphtholphthalein, ·1 gm. in 150 c.c. alcohol + 100 c.c. water: or better: Phenyl red ·02 per cent. solution in alcohol.

Phenolphthalein ·5 g.m dissolved in 1 litre of 50 per cent. alcohol.

Choice of a suitable indicator.

10 c.c. of about ·5 N. hydrochloric acid are taken in one test-tube and the same quantity of ·1 N. caustic soda in a second. To each is added the same volume, say 5 drops of methyl red. These solutions give us the colours which the indicator possesses at extreme acid and extreme alkaline reactions, respectively. The same test is made with all the other indicators. Five drops of each of the different indicators are now added to separate tubes each containing 10 c.c. of the fluid under investigation (in this case tap-water). There will be found some tubes in which the indicator has taken on the same tint as in either the hydrochloric acid or the caustic soda solution. These particular indicators are unsuitable for our purpose. But one tube at least will shew a colour intermediate between these extremes: the indicator contained in this is the one which must be used for the subsequent investigation. In our particular case neutral red or phenyl red will be found to be suitable.

The actual determination of h.

To 10 c.c. of the liquid under investigation a quantity of the chosen indicator is added such that the depth of colour is the most suitable for the colour matching—in our case 2 to 5 drops of neutral red. The indicator solution should be dropped in quite slowly from a uniformly-delivering pipette, and the exact number of drops must be carefully noted. In another test-tube a mixture of 5 c.c. of the above-mentioned secondary phosphate solution with an equal volume of the primary phosphate, is made, and to this is added exactly the same number of drops of the same indicator. By comparison of the colours it is determined whether the phosphate mixture is more acid or more alkaline than the unknown. With this information it is possible now to prepare another phosphate mixture which will be judged to give a nearer match with the

unknown when tested with the same indicator, for example, a mixture of 6 c.c. of secondary phosphate with 4 c.c. of primary phosphate, the total volume of the mixture being kept at 10 c.c. in all cases. This particular mixture may be called "phosphate mixture 6." In a similar way one prepares and tries other phosphate mixtures until the appropriate one has been discovered. This may be considered to be achieved when one solution which is a little too acid and a second which is slightly too alkaline have been obtained. Finally the intermediate mixture which gives a colour indistinguishable from that of the unknown is determined. As a rule, for fresh tap water this will be found to be phosphate mixture "8·7." The comparison of the colours should be carried out against a background of pure white writing paper placed at a distance of 10 cm., the tubes being viewed from above through the whole depth of the solution. Of course the tubes must be of equal diameters, and must be specially selected with regard to this requirement before the experiment. The pH of the unknown fluid is equal to that of the phosphate mixture which gives the same colour, and this is read off from a diagram constructed by Sörensen from the results of electrometric measurements. (fig. 2) This diagram refers also to other buffer mixtures: the method of using it is as follows:—

In order to find the pH of a mixture of 8·7 c.c. of secondary phosphate + 1·3 c.c. of primary phosphate, the value 8·7 is sought on the ordinate. The horizontal through this point cuts the curve labelled "phosphate" at a certain point whose abscissa is at a pH value of 7·60. This is about the value which is ordinarily obtained with tap-water.

Tap-water which has been allowed to stand is usually more alkaline on account of loss of CO_2, i.e. it shews a yellower tint with methyl red, and the phosphate mixture which gives the same colour is not "8·7" but "9" or even higher. Further it is easy to demonstrate the increase of alkalinity which occurs in tap-water when it is boiled for a short time.

The other buffer solutions whose pH values are recorded in the diagram are prepared from the following stock solutions:

"Glycocoll" indicates a solution of 7·505 gms. of glycocoll and 5·85 gms. of NaCl in 1 litre of water.

"HCl" is ·1 N. hydrochloric acid.

"NaOH" is a ·1 N. solution of caustic soda free from CO_2. It is

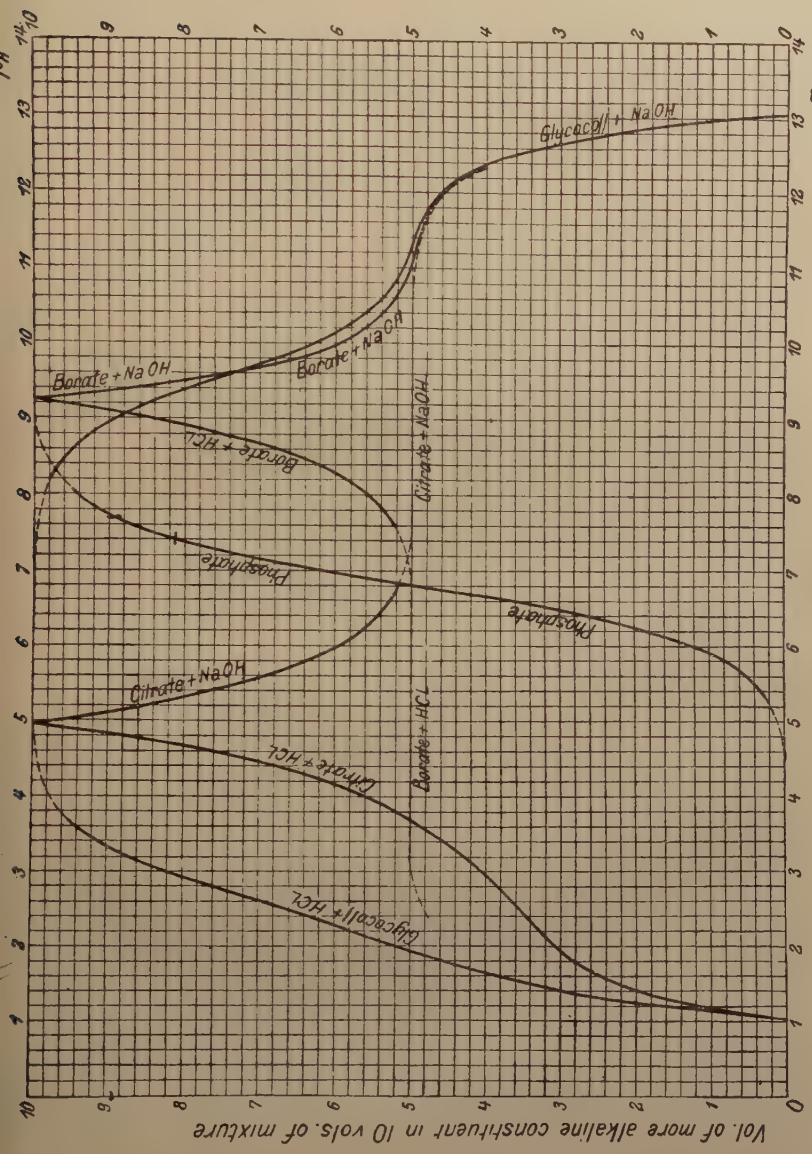

FIG. 2.—Sörensen's Buffer Diagram. The dotted portions of the curves are not reproducible with certainty and should not be employed.

prepared by adding caustic soda to water in a tall glass cylinder fitted with a greased stopper until the solution is saturated and an abundant insoluble residue remains. The mixture is well shaken several times and then allowed to stand for several days, or better, weeks, to settle. A portion of the clear upper fluid is then drawn off and diluted to the required strength, the above-mentioned precautions being taken against contact with CO_2. The solution so prepared is free from CO_2 because Na_2CO_3 is insoluble in the strong soda solution.

"Citrate" is a solution of 21·008 gms. of citric acid + 200 c.c. of N. caustic soda solution made up to 1 litre.

"Borate" is a solution of 12·404 gms. of boric acid and 100 c.c. of N. caustic soda made up to 1 litre.

All these solutions must be made up with CO_2-free water as already described, and must be stored out of contact with CO_2.

An example of the use of the diagram for these buffers: in order to find the pH of a mixture of 6 c.c. citrate + 4 c.c. hydrochloric acid (note that the total volume is always 10 c.c.) the point of intersection of the horizontal through the ordinate "6" with curve labelled "citrate + HCl" is sought; the abscissa of this point, namely 4·18 is the pH required.

The above-mentioned indicators cover only a limited range of reaction. The following is a selection of indicators useful for more diverse pH values.

Indicator	Colour change Alkaline—Acid	pH range for which applicable	Strength of solution employed
Tropæolin 00	yellow–red	1·4–2·6	·1% aqueous solution
Red cabbage extract	blue–red	2·0–4·5	500 gms. of chopped red cabbage treated with 500 c.c. of 96% alcohol for 2 days and then filtered.
Methyl orange	yellow–red	3·1–4·4	·1 gm. in 300 c.c. alcohol + 200 c.c. water.
Methyl red	yellow-red	4·2–6·3	
p-Nitrophenol	yellow-colourless	4·0–6·4	·1 gm. in 15 c.c. alcohol + 235 c.c. water
Neutral red	yellow–red	6·5–8·0	·1 gm. in 500 c.c. alc + 500 c.c. water
α-Naphtholphthalein	blue-green—greyish-yellow	7·3–8·7	·1 gm. in 150 c.c. alc. + 100 c.c. water
Phenolphthalein	red–colourless	8·3–10·0	·1gm. in 100 c.c. alc. + 100 c.c. water
Thymophthalein	blue–colourless	9·3–10·5	·1 gm. in 125 c.c. alc. + 125 c.c. water
Alizarin yellow R	red–yellow	10·1–12·1	·1% aqueous solution

Clark and Lubs[1] recommend for Sörensen's method the following excellent indicators which have very striking colour changes:—

Chemical name	Common name	pH range	Colour change	Concentration at which used in alcoholic solution
Thymolsulphonphthalein ..	Thymol blue	1·2–2·8	red–yellow	·04%
Tetrabromphenolsulphonphthalein ..	Bromphenol blue	3·0–4·6	yellow-blue	·04%
o-Carboxy-benzene-azo-dimethyl aniline ..	Methyl red	4·4–6·0	red-yellow	·02%
Di-brom-o-cresolsulphonphthalein ..	Bromcresol purple	5·2–6·8	yellow–purple	·04%
Di-brom-thymolsulphonphthalein ..	Bromthymol blue	6·0–7·6	yellow-blue	·04%
Phenolsulphonphthalein	Phenol red	6·8–8·4	yellow-red	·02%
o–Cresolsulphonphthalein	Cresol red	7·2–8·8	yellow-red	·02%
Thymolsulphonphthalein	Thymol blue	8·0–9·6	yellow–blue	·04%
o-Cresolphthalein	Cresolphthalein	8·2–9·8	colourless red	·02%

EXERCISE 14.

The Salt Error of Indicators.

Besides hydrogen ions, other ions also influence the colour of an indicator, but usually only when they are present in very high concentrations. In solutions rich in salts, therefore, the determination of pH by means of indicators is subject to a small error the magnitude of which depends on the particular indicator used and on the nature and concentration of the salt present. In the case of the indicators we have mentioned the error produced by salt solutions of physiological strengths is so small that it can usually be neglected. But as the "salt error" of indicators furnishes a very instructive theoretical introduction to the study of other important biological actions of salts, we will give an example of it.

In each of four tubes a mixture is made of 4·5 c.c. of M/15 primary phosphate and 4·5 c.c. M/15 secondary phosphate (as above); then to each tube further additions are made as follows:

Tube No.	1	2	3	4
Saturated KCl solution (about 3·5 M.)	0	1	10	1
Distilled water	1	0	1	0

[1] Lubs & Clark, *Journ. Washington Acad. Sci.*, **5**, 609, 1915, and Clark & Lubs, *Journ. Bacteriol*, **2**, 1, 1917, and particularly the valuable book *The Determination of Hydrogen Ions*, by Clark, 2nd Edn. 1922 (Baltimore, Williams and Wilkins).

Then a few drops of litmus solution (Kubel-Thiemann) are added to tubes 1 and 2 and a few drops of neutral red to tubes 3 and 4. On comparing the tubes containing litmus it is seen that their colours are almost identical, that to which the salt was added being slightly bluer than the other. From this we should conclude that the salt has produced at most a trifling diminution in the value of h i.e. a slight increase in the value of pH. On the other hand, when the tubes containing neutral red are compared it is found that that to which the salt was added is distinctly redder than the other ; i.e. the value of h appears to have become appreciably greater (pH smaller). The lack of correspondence between the results given by the two indicators leads us to doubt the validity of both.

If now we attempt to estimate the pH's by finding, as before, the phosphate mixture which gives the same colour, we find that phosphate mixture " 5 " gives a match with each of the tubes without KCl, so that the pH in both of these is 6·81.

In the tubes with KCl, however, we find that—

for litmus the pH comes to be pratically the same as before, namely 6·81.

for neutral red a match is obtained with phosphate mixture " 3·4 " so that the pH appears to be 6.35.

In order to solve this problem we must have recourse to an independent method of measuring h. If we apply the electrometric method (described later) to the solutions with and without the KCl we find that—

without salt pH=6·80
with salt pH=6·56

The result for the solution without salt thus agrees with that given by both indicators. It is obvious that this must be so, for the indicator method is standardised by parallel electrometric measurements on buffer solutions poor in salts. But with the solution containing the added salt, on the other hand, the electrometric method gives a value which is about mid-way between those given by the two indicators. It is theoretically justifiable to regard the electrometric value as " correct," and to ascribe any divergences shewn by indicators in solutions containing high concentrations of salts to " salt errors." These errors may be either positive or negative.

As Bjerrum has shewn, even the gas-chain in presence of tolerably high concentrations of electrolytes measures not the true concentration of hydrogen ions but a quantity which he calls the "hydrogen-ion activity," aH. According to the nature and quantity of the salts present, this is several per cent. smaller than the true concentration of hydrogen ions from which it may be calculated theoretically. The word "activity" is used in the same sense as "active mass" in dealing with the law of mass action—a quantity which similarly fails to shew an exact proportionality to the true concentration in highly concentrated solutions.

The chemical reactivity of the hydrogen-ions in the sense of the law of mass action as well as their electromotive activity, in general their "active mass," is thus not exactly proportional to their true concentration. It is this circumstance which justifies us in taking as standard the values obtained by the electrometric method. For it is just this chemical reactivity of the hydrogen-ions in a solution that we wish to determine by our measurements.

In conclusion let us repeat that at physiological salt concentrations this salt error is always very small. All the indicators we have mentioned have been selected with regard to the smallness of this error, i.e. we have chosen only those whose readings in solutions containing moderate quantities of salts or proteins agree most closely with those given by the electrometric method.

All these circumstances combine to reduce the uncertainty in the result of a measurement of pH by an indicator method to at most several units in the second decimal place.

EXERCISE 15.

The Protein and Alkaloid Errors of Indicators.

The indicator method involves the assumption that there is no substance present in the solution that will combine with the indicator and so, in addition to the hydrogen ions, play a part in determining its degree of dissociation. The most important of such substances are the proteins. The indicators we have recommended are all chosen for the smallness of their "protein errors" which in most cases can be entirely neglected under ordinary circumstances. There are other substances besides proteins which give rise to similar errors. The following will serve as an example:

Phosphate mixture No. "4" is prepared. To one 5 c.c. portion of this ·5 c.c. of distilled water is added, and to a second ·5 c.c. of 1 : 100 quinine hydrochloride. To each tube is then added about six drops of bromthymol blue. The resulting colours are very different, although it is quite impossible that the presence of so small a quantity of quinine should produce any appreciable change in pH in so heavily buffered a solution. On long standing a coloured compound of the indicator with quinine separates out in flakes. p-Nitrophenol (·2 c.c. of a 1 : 1000 solution) gives the same colour in these phosphate mixtures both with and without the quinine.

In the presence of such alkaloids pH estimations may be carried out with the single-coloured indicators of the nitrophenol series, but not with the indicators of Clark and Lubs.

EXERCISE 16.

The Estimation of Hydrogen-ion Concentrations by means of Indicators without Buffers.[1]

The method is most simply carried out with the so-called single-coloured indicators which change from a colourless form into a single coloured modification. Its principle is as follows: 10 c.c. of the fluid under investigation are treated with a measured quantity of the indicator solution. If the indicator is a suitable one it will neither be colourless nor will it take on the maximum depth of colour which it would shew in strongly alkaline solution. The indicators here employed have their maximum depth of colour in ·01 N. NaOH; stronger alkali produces no further deepening of the tint. By means of a serial experiment one determines how much of the indicator must be added to 10 c.c. of ·01 NaOH in order to produce the same depth of colour as has been produced in the unknown solution. The volume required will be smaller. We will call the ratio of this volume of indicator solution to that added to the unknown liquid the " colour index," C. This must always be less than 1. From its value the h of the unknown liquid may be calculated by means of the formula

$$h = k \cdot \frac{1-C}{C}$$

where k is a number characteristic of the particular indicator used—the " indicator constant."

Let us again, as in the previous exercise, take the case of the estimation of the h of fresh tap water. The appropriate indicator for the purpose is m-nitrophenol of which ·3 gm. should be dissolved in 100 c.c. of distilled water with gentle heating. 10 c.c. of the water to be investigated are taken in a test-tube and 1 c.c. of the indicator solution is added. After 2—3 minutes the indicator will have taken on its definite final tint. Meanwhile in each of three uniform test-tubes 9 c.c. are taken of a ·01 N. solution of NaOH which has been freshly made by diluting a 1 N. solution.

[1] L. Michaelis and A. Gyemant, *Biochem. Zeitschr*, **109**, 165. 1920.

(The exact strength of this NaOH solution is of no consequence—a ·02 N. solution could also be used.) A 10-fold dilution of the indicator solution with distilled water is now prepared, and of this ·5 c.c. is added to the first tube of caustic soda solution, 1 c.c. to the second, and 2 c.c. to the third, so that when all the three tubes of the series have been filled up with the ·01 N. NaOH solution to the same volume as is present in the tube of unknown solution—in this case, including the indicator, 11 c.c.—the respective concentrations of indicator form a geometrical series with the factor 2. On comparing the tubes in turn it will now be found that the first of the NaOH tubes is lighter than the unknown, the third is darker, while the middle one gives a fairly good match. In making this comparison it is necessary merely to hold the tubes together in a good light and not too far from a white background such as a sheet of writing paper or a porcelain dish. It is best to look from above down through the tubes, but observation from the side is also often advantageous.

The observation is now made more accurately by means of a finer series with factor 1·2 or even 1·15. In the rough experiment we found 1·0 c.c. of indicator solution to give the nearest match: we therefore now try 1·2 c.c. and 1·44 c.c. and also ·83 and ·69 c.c. of the diluted indicator.

The final result turns out to be that 1·0 c.c. is the correct amount, even 1·2 c.c. being too large and ·83 too small. Thus 1·0 c.c. of the original indicator solution in the unknown fluid produces the same depth of colour as 1·0 c.c. of the 10-fold diluted indicator in the same volume of caustic soda solution. The colour index is therefore ·10. In order to be able to apply the above formula we need yet to know the value of k for m-nitrophenol. At room temperature this amounts to $4{\cdot}7 \times 10^{-9}$ so that

$$h = \frac{1 - {\cdot}10}{{\cdot}10} \times 4{\cdot}7 \times 10^{-9} = 42 \times 10^{-9} = 4{\cdot}2 \times 10^{-8}.$$

To express this in pH notation:

$$\log.\ h = {\cdot}62 - 8 = -7{\cdot}38,$$
$$pH = 7{\cdot}38.$$

This value is found to vary within only narrow limits (about $\pm{\cdot}05$) provided that the tap water has not been kept long enough to lose an appreciable amount of its CO_2. This brings the determination to an end.

In the more general application of the method we calculate pH directly from the colour equation as follows:

Taking logarithms of both sides, we have:

$$\log. h = \log. k + \log.\frac{1-C}{C}.$$

from which,

$$pH = p_k + \log.\frac{C}{1-C}.$$

where p_k, the "indicator exponent" stands for the negative logarithm of the indicator constant. The following table gives the values of p_k for a few commonly employed indicators. They vary somewhat with temperature, but the method is applicable for pH estimations at any desired temperature once these values are known. From curve 1 in fig. 3 the value of $\log.\frac{C}{1-C}$ can be read off for any value of C that may be found. Values of C are plotted along the ordinate and values of $\phi = \log.\frac{C}{1-C}$ along the abscissa. As it is difficult to read values of the ordinate of less than ·1 the initial portion of the curve is reproduced with the ordinate unit magnified ten times (curve II) and also with the ordinate unit magnified 100 times (curve III). In using these curves the ordinate values must be divided by 10 or 100, respectively, while the abscissa values remain unchanged throughout.

Table 1. Summary of the Single-coloured Indicators.

Ordinary name	Chemical name	Colour	Pk at 18°	pH range for which applicable	Stock solution
β-Dinitrophenol	1–oxy–2,6 di-nitrobenzene	yellow	3·69	2·2–4·0	·1 gm. in 300 c.c. water
α-Dinitrophenol	1–oxy–2,4 di-nitrobenzene	yellow	4·06	2·8–4·5	·1 gm. in 200 c.c. water
γ-Dinitrophenol	1–oxy–2,5 di-nitrobenzene	yellow	5·15	4·0–5·5	·1 gm. in 200 c.c. water
p–Nitrophenol ..	p–nitro-phenol	yellow	7·18	5·2–7·0	·1 gm. in 100 c.c. water
m–Nitrophenol ..	m–nitro–phenol	yellow	8·33	6·7–8·4	·3 gm. in 100 c.c. water
Phenolphthalein	phenolphtha-lein	red	9·73	8·5–10·5	·04 gm. in 30 c.c. alcohol + 70 c.c. water
Alizarin yellow GG (salicyl yellow)	m–nitroben-zene–azo-salicylic acid	yellow	11·16	10·0–12·0	·05 gm. in 50 c.c. alcohol + 50c.c. water

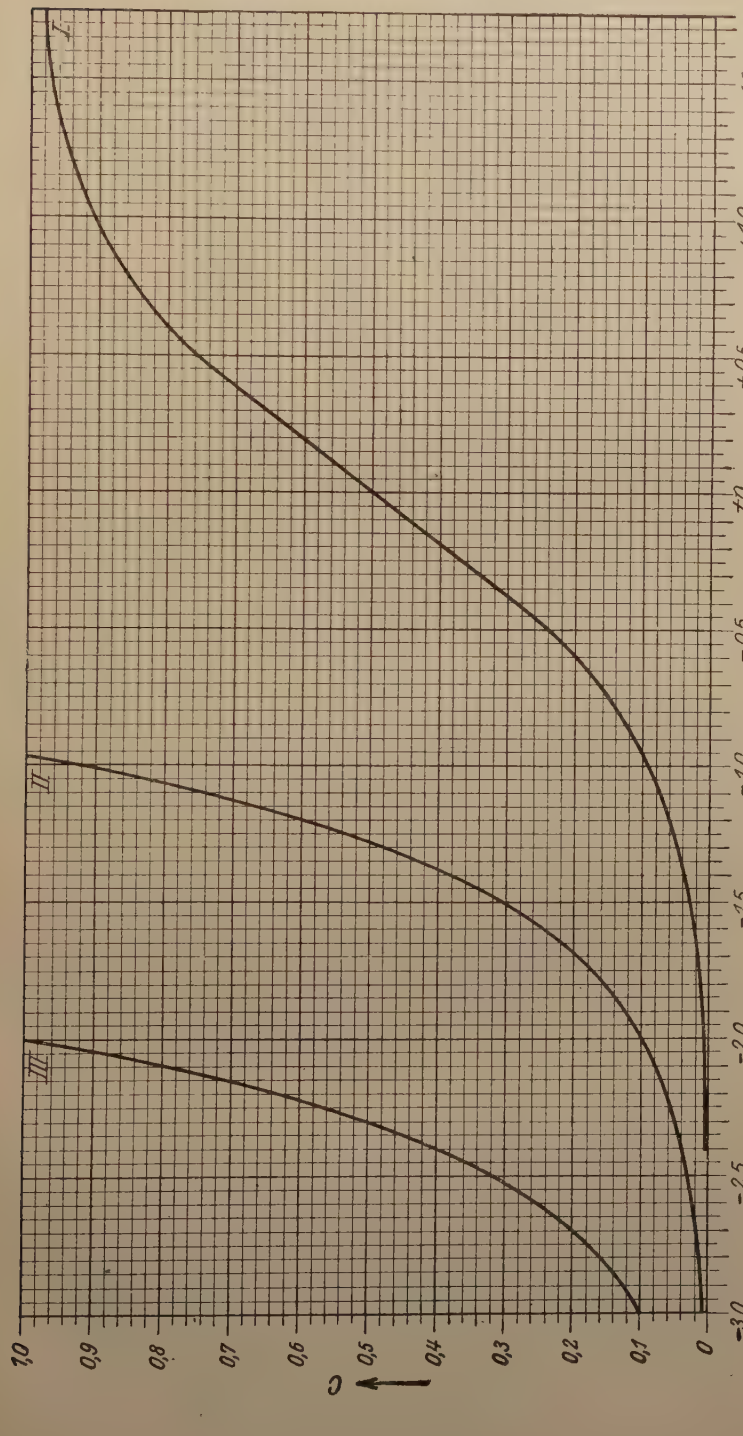

FIG. 3.—Diagram for the Indicator Method without Buffers.

Table 2. Indicator Constants p_k at various temperatures.

Temperature	β–Dinitro-phenol (1–2–6)	α–Dinitro-phenol (1–2–4)	αDinitro-phenol (1–2–5)	p–Nitro-phenol	m–Nitro-phenol
0°	3·79	4·16	5·24	7·39	8·47
5°	3·76	4·13	5·21	7·33	8·43
10°	3·74	4·11	5·18	7·27	8·39
15°	3·71	4·08	5·16	7·22	8·35
18°	**3·69**	**4·06**	**5·15**	**7·18**	**8·33**
20°	3·68	4·05	5·14	7·16	8·31
25°	3·65	4·02	5·11	7·10	8·27
30°	3·62	3·99	5·09	7·04	8·22
35°	3·59	3·96	5·07	6·98	8·18
40°	3·56	3·93	5·04	6·93	8·15
45°	3·54	3·91	5·02	6·87	8·11
50°	3·51	3·88	4·99	6·81	8·07

All these indicators change from yellow in alkaline solution to colourless in acid solution, except phenolphthalein which changes from red to colourless.

The corresponding values of C and pH at room temperature (18°C.) for phenolphthalein and m-nitrobenzene-azo-salicylic acid are given in the following tables:

Phenolphthalein

C	pH	C	pH	C	pH
·010	8·45	·16	9·10	·55	9·80
·014	8·50	·21	9·20	·60	9·90
·030	8·60	·27	9·30	·65	10·0
·047	8·70	·34	9·40	·70	10·1
·069	8·80	·40	9·50	·75	10·2
·090	8·90	·45	9·60	·80	10·3
·120	9·00	·50	9·70		

m-Nitrobenzene-azosalicylic acid

C	pH	C	pH
·13	10·00	·56	11·20
·16	10·20	·66	11·40
·22	10·40	·75	11·60
·29	10·60	·83	11·80
·36	10·80	·88	12·00
·46	11·00		

Using these tables, the above example would be calculated as follows: The formula that is used is—

$$pH = p_k + \phi$$

From table II, p_k for m-nitrophenol at the temperature at which the experiment was carried out (18°C.) is 8·33. From the diagram on p. 45 it is seen that the value of ϕ corresponding to the observed colour index of ·10 is $-·95$ so that $pH = 8·33 - ·95 = 7·38$.

In order to shew that this method without buffers gives the same result as the indicator method using standard buffer solutions, measure by the two methods a solution made by mixing 20 c.c. N. NaOH with 21 c.c. N. acetic acid and diluting with distilled water to 200 c.c. The indicators to be used are methyl red and p-nitrophenol, respectively. The results should agree within the prescribed limits of error, namely within a few units in the second decimal place of the pH value.

EXERCISE 17.

The Acid-Error of Indicators.[1]

The indicator method can only give correct results if the pH of the fluid under investigation is not altered by the addition of the indicator. If, as is commonly the case, the indicator is either an acid or a base this condition will only be satisfied if the nature of the fluid is such that it is sufficiently well buffered to prevent such changes of reaction as the indicator might otherwise cause. For this reason it is impossible to measure the pH of absolutely pure distilled water by means of indicators, for this must necessarily be altered by the addition of the indicator. The method is more applicable to the case of ordinary distilled water which contains CO_2; but even this does not lend itself to the obtaining of exact results. River- and sea-water, in consequence of the bicarbonate and free CO_2 that they contain, are still better buffered. For these, intensely coloured indicators such as neutral red or phenol red can be used without special precautions because only the smallest quantities are required; but the indiscriminate use of the more feebly coloured indicators of the nitrophenol series may easily lead to errors which are known as the acid errors of the indicators. The following example will serve to demonstrate this: the pH of a sample of fresh tap-water is first measured by the method described on pp. 42–43. The result will probably lie between 7·3

[1] L. Michaelis and R. Krüger, *Biochem. Zeitschr*, **119**, 307. 1921; L. Michaelis, *Zeitschr. f. Unters d. Nahrungsmittel* **42**, 75. 1921.

and 7·4. A further sample of the same water when examined by the indicator method using standard buffers with neutral red as indicator will give a result between 7·5 and 7·6. It is therefore necessary to modify the indicator method without buffers so that the difference between the two methods disappears. This can be done by using a much smaller quantity of indicator than is recommended on p. 43, and comparing the colours by observing a greater depth of solution. It is convenient to use for the purpose, colourless test-tubes 25 cms. in height and capable of holding rather more than 40 c.c. They are held in the stand illustrated in fig. 4. In order to carry out a measurement with tap—or river—water a series of six such tubes is taken containing respectively ·25, ·29, ·33, ·38, ·45, and ·50 c.c. of a ten-fold dilution of the stock solution of ·3 gm. m-nitrophenol in 100 c.c. water (the same strength as previously used). To each tube there are now added 40 c.c. of N/50 NaOH freshly prepared by diluting the 1 N. solution—the exact strength being of no consequence, as before. 40 c.c. of the fresh tap-water are now taken in a further similar test-tube and m-nitrophenol is added until the colour is within the range covered by the six alkaline comparison solutions. Some 2—2·5 c.c. of the ten-fold dilution are ordinarily required for tap-water, and about 1 c.c. for sea-water. The indicator is uniformly distributed through the liquid by repeated inversion of the tube, but not, in the case of the tap-water, by pouring from one tube to another, as this might entail loss of CO_2.

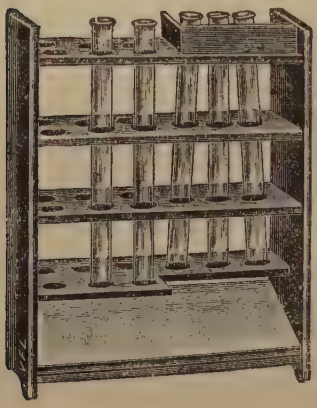

FIG. 4.

Three of the holes of the stand are separated off in a small frame; in the middle one of these is placed the tube of water under investigation and on either side a comparison tube. These are observed from above through the length of the solution against a sloping surface of opal glass that is illuminated by diffused daylight.

In this way the estimation is free from the error. In fact, so exact is it that it is appropriate to introduce the small corrections

for temperature and for small changes of salt content. The calculation of the exact pH is made by means of the formula:

$$pH = p_k + s + t + \phi$$

p_k is a constant characteristic of m-nitrophenol, and possesses the invariable value of 8·33.

s is the salt correction. For tap, river, and other practically salt-free waters, s = O. For sea-water of all concentrations that are likely to occur, s = −·16.

t is the temperature correction. The temperature of the water is measured directly in the tube, and the appropriate value of t is inserted from the following table:

Temperature in °C.								
5	10	15	17·5	20	25	30	35	40
t = +·10	+·06	+·02	±0	−·02	−·06	−·11	−·15	−·18

ϕ is read off from the diagram on p. 45.

In this way the pH of Berlin tap-water is usually found to be 7·6—a value identical with that obtained by Sörensen's method using neutral red as indicator, but somewhat greater than that (7·4) obtained by using m-nitrophenol without standard buffers in the way described in exercise 16, in which we used tap-water partly for the sake of simplicity, and partly in order that that exercise might serve as an introduction to the present one. In the case of the fluids that most commonly come up for investigation such as urine, bacteriological culture medium, beer, etc., there is no question of such an acid error for these fluids are well buffered by phosphates, lactates, proteins, etc.

The method described in this section is particularly suitable for use on scientific expeditions for the examination of sea and river water, because it does not involve the transport of the stock buffer solutions.

EXERCISE 18.

Measurement of the pH of a Coloured or Turbid Fluid by Walpole's Modification of the Indicator Method.

Suppose we wish to determine the reaction of normal acid urine by the indicator method without buffers. The need at once arises for avoiding the difficulties caused by the colour of the urine itself. For this purpose the Walpole principle is employed. The

simple piece of apparatus which is required is termed a comparator and is illustrated in fig. 5. It consists of a block of wood in which are bored six holes to receive test-tubes and three peep-holes a, b, c, through each of which a pair of test-tubes, standing one behind the other can be viewed simultaneously. At the back provision is made for the insertion of screens of frosted and of bright blue glass.

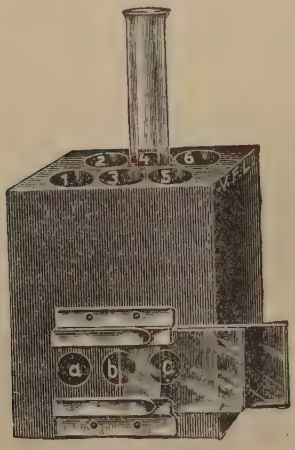

FIG. 5.—Comparator with three apertures, seen from behind.

In order to reduce the intensity of its colour the urine is diluted two or three times. The best diluting fluid is an approximately 2 per cent. solution of NaCl which corresponds roughly to the salt content of the urine, but it makes little difference if distilled water is used for the purpose. 10 c.c. of the diluted urine are measured into a test-tube, and an exactly measured quantity of the above-described stock solution of p-nitrophenol is added. As a rule, ·5—1 c.c. will be required to impart the necessary distinct but not too intense yellow colouration. The tube is now placed in hole No. 3 of the comparator. A second tube filled in exactly the same way but with an equal quantity of water instead of the indicator solution is placed in hole No. 1. In hole No. 4 is placed a tube of plain water. A series of dilutions of p-nitrophenol in N/50 NaOH, each having the same total volume as the mixture of urine and indicator, is now prepared as described in exercise 16. The problem now is to determine which of these dilutions when placed in hole No. 2 gives a match of the colours observed through peep-holes a and b. Unless the remaining peep-hole is used for a second comparison tube it is closed with the thumb. As the third hole can thus be dispensed

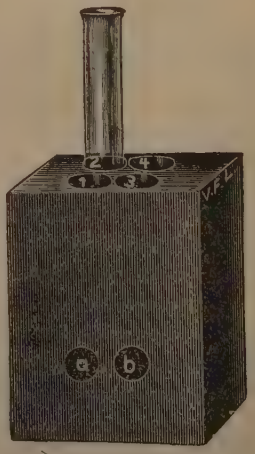

FIG. 6.—Comparator with two apertures seen from the front.

with, a two-holed comparator can also be used (fig. 6). The face of the comparator depicted in fig. 5, namely that which carries the frosted and blue screens, is the one which is directed towards the sky: the tubes are observed through the holes in the opposite face. Through both holes the combined colour of the indicator and of the urinary pigment is seen: in the one case these colours are actually mixed, being present in the same fluid; in the other case they are in separate fluids but are mixed optically, as the observation is made through both tubes placed one behind the other. Thus the two colours are combined similarly in both cases, and equality of colour can be produced only when the indicator possesses the same depth of colour in both tubes. The blue screen is used to convert the various intensities of yellow into different tints ranging from yellow through green to blue; this makes the colour matching easier. With indicators of other colours the blue screen is omitted and only the frosted glass is used. The calculation of pH is made as in exercise 16. For example it might be found that normal urine containing ·75 c.c. of the p-nitrophenol solution gave a match with the alkaline solution containing ·30 c.c. of the 10-fold diluted indicator. The colour index is therefore

$$\frac{\cdot 030}{\cdot 75} = \cdot 040 \text{ so that } \mathrm{pH} = 7 \cdot 16 - 1 \cdot 38 = 5 \cdot 78.$$

The pH of urine is usually between ·5 and 7.

The Walpole principle is of similar value for the investigation of turbid fluids, such as a nutrient broth containing bacteria. Fresh broth usually has a pH of 7 – 7·5 (m-nitrophenol as indicator); glucose broth inoculated with Bacterium coli attains a pH of about 5 (indicator γ-dinitrophenol).

This comparator[1] was originally intended for the estimation of the pH of coloured fluids by Sörensen's method (exercise 13) but it serves equally well for the method without buffers.

EXERCISE 19.

On the Theory of the Colour Changes of Indicators.

Wilhelm Ostwald explains the colour changes of indicators by assuming that the ions of these substances possess a colour different from that of the undissociated molecule. Hantzsch, however,

[1] Comparator of Hurwitz, Meyer and Ostenberg, *Proc. Soc. Exp. Biol. Med.* **13**, 24. 1915.

shewed that the colour change is accompanied by a tautomeric re-arrangement of the molecule (from a quinoid to a lactoid form, for example). These opposing theories are reconciled in the modern views on molecular structure, according to which the appearance of a free electric charge as the result of ionisation would suffice to produce a re-arrangement of the internal structure of a molecule that is liable to tautomeric change. Such a tautomeric change will not necessarily take place suddenly—indeed, cases are known in which several hours are required for the completion of the visible colour change, although the preliminary ionisation must have taken place instantaneously. Acid fuchsin and water-blue furnish good examples of this behaviour. Three phosphate mixtures of pH 6·5, 7·0, and 7·5, respectively, are prepared, and to 10 c.c. of each of these a suitable quantity of a 1 per cent. solution of water-blue is added. At first all three tubes shew the same depth of colour, but later they gradually fade—the more completely the higher the pH value of the particular solution—until after several hours each tube has taken on its definitive final tint. The colour change is more rapid at higher temperatures. An indicator that behaves in this way would, as a rule, be unusable in practice.

EXERCISE 20.

Simplification of the Indicator method without Buffers. Permanent Indicator Series.

The single-coloured indicators of the nitrophenol series keep practically indefinitely in solution. It is therefore not necessary to prepare the comparison solutions afresh for each determination; they can be stored ready for use in sealed tubes. The necessary solutions have the following compositions:

The following stock solutions are first prepared:

m-nitrophenol	..	·3 gm. in 100 c.c. distilled water
p-nitrophenol ..	..	·1 gm. in 100 c.c. ,, ,,
γ-dinitrophenol	..	·1 gm. in 400 c.c. ,, ,,
α-dinitrophenol	..	·1 gm. in 200 c.c. ,, ,,

For the preparation of the permanent indicator series portions of the stock solutions are diluted exactly ten times (2 c.c. + 18 c.c. distilled water, for example). Several sets of similarly calibrated

test-tubes with drawn out necks (" sealing tubes ") are then prepared and into these tubes are measured the several quantities of the 10-fold dilution of the indicator mentioned in the following table. ·1 N. sodium carbonate solution is added to each tube to bring up the volume to exactly 7 c.c. and then it is sealed off and labelled with its appropriate pH value as given in the table.

I. Permanent series for m-Nitrophenol.

Tube No.	1	2	3	4	5	6	7	8	9
c.c. Indicator	5·2	4·2	3·0	2·3	1·5	1·0	·66	·43	·27
pH Label ..	8·4	8·2	8·0	7·8	7·6	7·4	7·2	7·0	6·8

II. Permanent series for p-Nitrophenol.

Tube No.	1	2	3	4	5	6	7	8	9
c.c. Indicator	4·05	3·0	2·0	1·4	·94	·63	·40	·25	·16
pH Label ..	7·0	6·8	6·6	6·4	6·2	6·0	5·8	5·6	5·4

III. Permanent series for γ-Dinitrophenol.

Tube No.	1	2	3	4	5	6	7	8
c.c. Indicator	6·6	5·5	4·5	3·4	2·4	1·65	1·1	·74
pH Label ...	5·4	5·2	5·0	4·8	4·6	4·4	4·2	4·0

IV. Permanent series for α-Dinitrophenol.

Tube No.	1	2	3	4	5	6	7	8	9
c.c. Indicator	6·7	5·7	4·6	3·4	2·5	1·74	1·20	·78	·51
pH Label ..	4·4	4·2	4·0	3·8	3·6	3·4	3·2	3·0	2·8

It is not worth while to add a ninth tube to series III. as its colour would be too faint.

The colours of the last tubes of the series IV. are also very weak; these tubes are advantageously replaced by a short series made up with β-dinitrophenol. The stock solution of this indicator is ·1 gm. in 300 c.c. of distilled water, and as in the other cases this is diluted ten times for the preparation of the permanent series.

V. Permanent series for β-Dinitrophenol.

Tube No.	1	2	3	4	5
c.c. Indicator ..	2·44	1·68	1·15	·76	·49
pH Label	3·2	3·0	2·8	2·6	2·4

The four permanent series are stored in the dark in the stand represented in fig. 7; they keep practically indefinitely. The pH values hold for the following method of use: Into tube No. 1

of the comparator 6 c.c. of the liquid under investigation are measured, and 1 c.c. of the undiluted stock solution of the appropriate indicator is added; tube No. 2 receives 6 c.c. of the liquid under investigation +1 c.c. of water. Tube No. 3 contains pure water only. It is now necessary to determine which tube from the corresponding indicator series must be placed in hole No. 4 in order that the observed colours shall match when viewed through the frosted and blue screens. The pH intervals in these series have the value ·2, and intermediate values can easily be estimated so that the maximum possible variation in the result may be taken as ±·05, provided that no salt or protein errors have to be considered. As a matter of fact, these particular indicators all possess very small salt and protein errors.

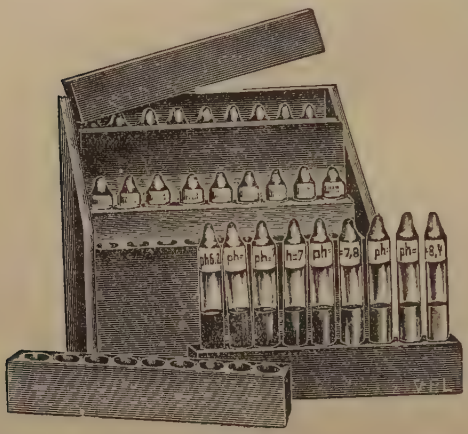

FIG. 7.—Case for the Permanent Indicator Series covering the range pH 2·8 to 8·4.

Very strongly coloured fluids, provided that they are sufficiently well buffered, can be diluted three times without influencing the accuracy of the estimation and even, when necessary, as much as ten times without appreciable error. Distilled water will serve as the diluting fluid. The following materials will serve for practice with the method: for m-nitrophenol, nutrient broth (diluted three times): for m- or p-nitrophenol, human urine (diluted 2–3 times): for γ-dinitrophenol, light beer (diluted three times): dark beer (diluted 5–10 times), glucose broth containing a 24-hours' growth of Bacterium coli: gastric juice of a suckling (diluted 2–3 times, if necessary).

A further example is 10 per cent. gelatin solution. It is melted on the water bath, diluted, if necessary, and treated with 1 c.c. of indicator solution or water, as the case may be, and then allowed to cool. The solidification makes no difference; the procedure is as usual. Solid agar nutrient media may be dealt with in the same way.

EXERCISE 21.

The difference between True Acidity and Titration Acidity.

The true acidity of a solution is its hydrogen-ion concentration. The titration acidity is the measure of its power to combine with alkali. In the case of the titration of strong acids such as HCl HNO_3, and H_2SO_4 the true acidity and the titration acidity are proportional to each other. Further it is of little consequence which indicator is used for the titration. Suppose that 10 c.c. of ·1 N. HCl is titrated with ·1 N. NaOH. and that the indicators used in three parallel experiments are respectively:

(a) Phenolphthalein (the solution recommended on p. 38). The titration is carried on until the appearance of the faintest rose-red colour.

(b) Litmus tincture (Kubel-Tiemann). The titration is at an end when a violet colour is produced.

(c) Methyl orange (the solution described on p. 32). The titration is at an end when the red tint has completely disappeared, i.e. when the next drop of NaOH solution produces no further change in the weak yellow colour.

The three results will be identical; if their value is exactly 10 c.c. the strength of the acid must have been exactly ·1 N. The concentration of hydrogen-ions in the acid will also be practically exactly ·1 N. if we disregard the incompleteness of the ionisation.[1]

If, on the other hand, we titrate 10 c.c. of ·1 N. acetic acid with ·1 N. NaOH we find that

(a) with Phenolphthalein, 10 c.c. will be required.

(b) with Litmus almost as much.

(c) Methyl orange begins to turn yellow when 4—5 c.c. of alkali have been added, but so gradually that no definite end point can be assigned to the titration; even the expected end point with 10 c.c. of NaOH is not to be observed.

If, lastly, we measure the h·of the acetic acid solution by the method given on p. 52 we obtain a value of about $1{\cdot}4 \times 10^{-3}$. Here

[1] Or rather, the diminution of the H-ion activity as defined by Bjerrum, see p. 32.

the hydrogen-ion concentration by no means coincides with the titration acidity; and the latter even varies according to the indicator which is used.

During the titration the h changes step by step with the addition of the successive quantities of alkali. The end point for phenolphthalein marks the point at which the pH value passes through 8, the end point for litmus the transition point through pH 7, approximately, and for methyl orange the change through pH 5.

In the titration of a strong acid these points follow one another so rapidly that it matters little which indicator is used. But in the case of acetic acid the change from pH 6 to pH 8 is brought about only by the addition of a large quantity of soda, so that the choice of indicator is of importance.

Thus the value of h can never be determined by titration except in the case of a strong acid. The process of titration can, however, be used to achieve one of two objects:

(a) To determine how many c.c. of alkali are required to produce an exactly neutral solution. In this case, of the three indicators mentioned, only litmus can be used. The end point can often be determined only roughly because the pH changes only slowly as the alkali is added, so that the colour change is quite gradual. The determination of this point has, however, very little practical importance.

(b) To determine the number of equivalents of acetic acid that are present in the solution. In this case the caustic soda must be added until a pure solution of sodium acetate is produced so that the next drop of alkali is in excess. The indicator must therefore mark the point at which the h is the same as in a pure solution of sodium acetate. As this salt in consequence of hydrolytic dissociation is slightly alkaline (pH between 7 and 8, according to the concentration) phenolphthalein is the indicator to be chosen; the point at which it just begins to shew a red tint marks a pH value of 8.

Examples of solutions having the same h but different titration acidities:

1. ·0014 N. HCl and ·1 N. acetic acid have the same h, about $1{\cdot}4 \times 10^{-3}$. But the titration acidity against ·1 N. caustic soda, using phenolphthalein as indicator, is hardly measurable for the HCl (71·4 c.c. being neutralised by 1 c.c. of the alkali) while 10 c.c. of the acetic acid require 10 c.c. of the soda.

2. A mixture of 20 c.c. N. acetic acid and 10 c.c. N. NaOH is prepared, and also a 10-fold dilution of this mixture. In both solutions h has the same value (see p. 32, c) amounting to about 2×10^{-5}. But 10 c.c. of the first solution titrated against ·1 N. NaOH using phenolphthalein require 33 c.c. of the alkali, while 10 c.c. of the second solution require only 3·3 c.c.

The titration of an aqueous solution of phosphoric acid is very instructive. A solution of primary sodium phosphate, NaH_2PO_4, reacts just acid to methyl orange; one drop of alkali in excess changes the colour to bright yellow. Secondary sodium phosphate, Na_2HPO_4, on the other hand, just reacts distinctly alkaline to phenolphthalein, so that a single drop of acid completely discharges the colour. Tertiary sodium phosphate, Na_3PO_4, reacts strongly alkaline and behaves like caustic soda to phenolphthalein. If now phosphoric acid is titrated against ·1 N. NaOH using a little methyl orange and phenolphthalein simultaneously as indicators, just twice as much alkali will be required to arrive at the turning point of phenolphthalein as at that of the methyl orange. The correct end point for methyl orange is that at which a trace of the orange colour is still visible but is changed to a pure pale yellow on the addition of a single extra drop of the caustic soda solution. This "extra" drop is not to be included in the reading. The end point for phenolphthalein is reached by that drop of alkali which produces not merely a suggestion of pink but a full red colour; this last drop is included in the reading. The experiment can be carried out most clearly by titrating separately a diluted solution of primary potassium phosphate (see p. 33) using methyl orange, and a solution of secondary sodium phosphate (p. 34) using phenolphthalein. The end-points can be estimated to 1—2 drops.

EXERCISE 22.

The Titration of Gastric Juice.[1]

By the titration of gastric juice approximate answers can be obtained to two questions:

1. What is the value of h in the gastric juice? The importance of this question lies in the circumstance that pepsin will display its maximum activity only within a very restricted range of

[1] L. Michaelis, *Biochem. Zeitschr*, **79**, 1. 1917.

hydrogen-ion concentration, namely between pH 1·7 and pH 2. This is merely another way of asking: What quantity of "free HCl" is present?

2. How much HCl has the stomach produced altogether? —the "total HCl." As the stomach contains substances such as albumin and peptone that have the power of combining with acids not all the hydrochloric acid that is secreted remains "free."

The first question can be answered with sufficient exactness for practical purposes by means of a pH determination carried out by one of the methods already described using the Walpole principle. A more exact answer is to be obtained by using the hydrogen electrode (described later). An approximate result can be obtained by the following titration method whose validity has been established empirically by direct comparison with pH measurements:

10 c.c. of the filtered undiluted gastric juice are measured into a white porcelain dish and two drops of a ·1 per cent. alcoholic solution of dimethylaminoazobenzene are added. Owing to the presence of free hydrochloric acid the solution turns rose-red. The liquid is now titrated with ·1 N. alkali until a suspicion of an orange colour is seen, i.e. until the mixture is roughly salmon-coloured. This point is not exactly defined, but it can always be estimated to 2 or 3 drops. A full orange colour indicates an overshooting of the mark. This end point marks the neutralisation of the free HCl. If, for example, 3 c.c. of ·1 N. alkali have been required this means that 10 c.c. of the gastric juice contain as much free HCl as 3 c.c. of the ·1 N. acid. In the usual nomenclature of the subject it would be said that the "free" acidity is 30 (the number of c.c. of ·1 N. alkali required for 100 c.c. of the juice). The concentration of free HCl is therefore ·03 N., and this will also be the concentration of hydrogen-ions, if we assume, as we are justified in doing, in an experiment of this degree of accuracy, that the ionisation is complete. The pH therefore comes to be 1·5.

The total HCl is arrived at in the following way: to the same liquid there are added now (or at the beginning, if preferred) two drops of phenolphthalein, and the titration is continued until the dimethylaminoazobenzene shows a pure lemon yellow colour without admixture with orange, and the next drop of alkali produces no further change of colour. The reading (not including this last drop) is noted, and the titration continued still further until the red colour indicating the phenolphthalein end-point is observed.

The reading is once more noted, and the mean of this and the preceding reading is taken as the end point for the total HCl. For example; suppose that the readings for 10 c.c. of the gastric juice are:

To the salmon colour with dimethylaminoazobenzene 3·0 c.c.
,, pure yellow ,, ,, ,, 5·0 ,,
,, red colour with phenolphthalein .. .. 7·4 ,,

Then the end of the titration of the free HCl is at 3·0 c.c.
and the end of the titration of the total HCl is at 6·2 c.c.
and h = ·030 N.

Expressing the result in the nomenclature ordinarily adopted for the gastric juice we should say that:

Free HCl =30
Total HCl =62
Combined HCl=32

A specimen of gastric juice that at the outset gives a salmon or even orange or yellow colour with dimethylaminoazobenzene would be said to contain "no free HCl." The expression "lack of free HCl" cannot be defined with objective exactness; the above arbitrary definition is given merely for practical convenience.

If the gastric juice contains lactic acid, its acidity will be included in the "total HCl." The titration of the total HCl is therefore possible only in the absence of any significant quantity of lactic acid.

The values for very small quantities of total HCl (acidities of 10 or under) are not strictly accurate: in these cases the assumptions involved in the method are uncertain. But it suffices for clinical purposes.

V.

Optimum Precipitation Concentrations at various Reactions.

The principle of Serial values of h with constant Salt Concentration.[1]

The influence of hydrogen-ions on any solution can be investigated by means of a serial experiment in which there is used a set of suitable buffers so chosen that their several values of h form a geometrical series. This necessarily means that the corresponding pH values form an arithmetical series (see p. 29). But other ions, in addition to those of hydrogen, produce changes of greater or lesser extent in the condition of substances in solution, so that if the effects due to the hydrogen-ions alone are to be observed, the concentrations of other ions must be kept constant throughout each individual series. The problem thus resolves itself into the preparation of a series of solutions of increasing values of h in which the concentrations of all the other constituents remain unaltered. From the nature of things, it is impossible to fulfil this condition with mathematical exactness, for a change in the concentration of the hydrogen-ions can only be brought about by producing changes in the concentration of the other ions with which they are in equilibrium. But the problem can be solved approximately with a degree of exactness that suffices for all practical purposes in the following way: In a series of sodium acetate-acetic acid mixtures let us vary the amount of acetic acid while keeping the concentration of sodium acetate constant in all (not vice versa). Each of these solutions will now contain (1) Na'-ions; (2) acetate-ions; (3) H'-ions and (4) OH'-ions, which latter, however, do not call for separate consideration, as their quantity is always determined by that of the H-ions (see p. 28). Since the quantity of sodium acetate is kept constant, the concentration of sodium ions must also necessarily be constant, and the concentration of acetate ion will only increase as a result of the variation in the quantity of acetic acid present. But as in each case the acetic acid will be ionised to a very small extent

[1] L. Michaelis and P. Rona, *Biochem. Zeitschr*, **27**, 38. 1910.

the amount of acetate ion arising from the acid will be negligible compared with the relatively large amount produced from the well ionised sodium salt, so that the total concentration of acetate ion may also be regarded as sensibly constant. Thus, apart from the H-ions, the only substance whose concentration will vary throughout the series will be the undissociated acetic acid. But this carries no electric charge, and so in most cases is to be regarded merely as an indifferent substance. This is the way in which the problem of varying the hydrogen-ion concentration alone is solved in practice.

EXERCISE 23.

The Optimum Reaction for the Crystallisation (Point of Minimum Solubility) of m-Aminobenzoic acid.[1]

The point of minimum solubility, i.e. the optimum reaction for the crystallisation of an amino-acid occurs at its iso-electric point. The definiteness of this optimum depends on the value of the product $K_a \times K_b$, where K_a is the acidic dissociation constant and K_b the basic dissociation constant of the amino-acid. The highest values of $K_a \times K_b$ that are met with hardly exceed 10^{-16}, but in these cases the crystallisation optimum is fairly well marked. In cases where the value of $K_a \times K_b$ is smaller the optimum point is not so obvious. An amino acid which serves for the demonstration of a fairly sharp crystallisation optimum is m-aminobenzoic acid for which $K_a = 1{\cdot}6 \times 10^{-5}$ and $K_b = 1{\cdot}2 \times 10^{-11}$, so that $K_a \times K_b =$ about 2×10^{-16}. The iso-electric point, I, that is, the value of h at which the concentration of un-ionised amino-acid molecules is a maximum, is related to these dissociation constants in the following way:

$$I = \sqrt{\frac{K_a}{K_b} \cdot K_w}$$

where K_w is the ionisation constant for water, and has the value $\cdot 6 \times 10^{-14}$ at room temperature.

The value of I for m-aminobenzoic acid is therefore approximately:

$$I = \sqrt{\frac{1{\cdot}6 \times 10^{-5}}{1{\cdot}2 \times 10^{-11}} \times \cdot 6 \times 10^{-14}} = 9 \times 10^{-5}.$$

[1] L. Michaelis and H. Davidsohn, *Biochem. Zeitschr*, **30**, 143. 1910.

In order to demonstrate this an experiment is carried out as follows:

First the following mixtures are made up:

Tube No.	1	2	3	4	5
Water c.c.	2·5	2·4	2·2	1·8	1·0
7·5 N. acetic acid c.c.	·1	·2	·4	·8	1·6

To each of these tubes is now added 1 c.c. of an 8 per cent. solution of m-aminobenzoic acid in 1 N. NaOH. The tubes are next placed in a hot water bath for a few minutes, and then withdrawn and allowed to cool to room temperature, when the amino-acid gradually crystallises out in some such way as the following:

	1	2	3	4	5
The crystallisation is distinct	never	never	after 3 mins.	after 1 min.	never

At the end of the experiment the crystals can be re-dissolved on the water bath, and the observation confirmed repeatedly. The optimum crystallisation point is seen even more distinctly if the contents of each tube are diluted with 1 c.c. of water: it occurs in tube No. 4 which contains ·8 c.c. of 7·5 N. acetic acid + 1 c.c. N. NaOH, i.e. 5 equivalents of acetic acid to 1 equivalent of sodium acetate. The value of h for this solution is 1×10^{-4} while for that in tube 3 $h = \cdot 5 \times 10^{-4}$. These numbers are in good agreement with the calculated value of the iso-electric point.[1]

The calculation of the values of h or pH from the composition of the acetate buffer is only approximate because the presence of the amino-acid itself has not been taken into account. In order to fix the exact numerical value of the optimum crystallisation reaction one must not be content to calculate the values of h, but must measure them in each individual solution—preferably

[1] The instructions given in the first edition of this book for the performance of this experiment referred to a preparation obtained from Kahlbaum during the war and incorrectly labelled "m-aminobenzoic acid." On account of its small solubility and ease of crystallisation it served even better than the correct substance for the purpose of this demonstration, and apparently it had the same iso-electric point. It would be very welcome if some colleague could ascertain what this preparation really was: unfortunately no specimens are left for analysis. The above example is taken from L. Michaelis and H. Davidsohn, l.c. The preparations of m-aminobenzoic acid which are at present on the market are in general so impure that they require to be re-crystallised from hot water with the addition of abundance of animal charcoal before they are usable.

by means of the electrometric method described on a later page. For the purposes of this elementary demonstration, however, we can spare ourselves this trouble, because (1) at the iso-electric point itself the amino-acid can exert, theoretically, no influence at all on the h of the solution, and (2) in this particular experiment the differences between the true and the calculated values of h, even in the most unfavourable instances, are less than the h-interval between one tube and the next.

This approximate calculation shall serve for our purposes, but it must be remembered that for research purposes measurements of the h value in each tube would be indispensible if the results are to have any claim to absolute exactness. This is likewise true of all the remaining experiments we shall describe.

It can be imagined what a loss of the substance is experienced if a compound like aminobenzoic acid is recrystallised at a reaction other than the optimum.

EXERCISE 24.

The Precipitation Optimum for Casein at various Reactions.[1]

Just as there exists a particular reaction of minimum solubility for aminobenzoic acid so in an exactly analogous fashion there exists an optimum reaction for the precipitation of a colloidal protein. We shall illustrate this first of all for casein. The phenomenon can be explained in two ways which, however, are not mutually contradictory, but express the same principle in different language.

1. We can say that pure casein is insoluble, but that the casein-ions will dissolve whether they happen to be positively charged, as in strongly acid solutions, or negatively charged, as in alkaline solution. Consequently there must be a particular value of h at which, as in the case of aminobenzoic acid, there will be the minimum number of ions present and so a minimum of solubility.

2. Or we can regard a casein solution as a colloidal solution in which the disperse phase consists of aggregates of casein molecules. The electric charge on this disperse phase will depend on the concentration of hydrogen-ions (see the section "electrophoresis"); at a certain value of h, namely the iso-electric point of casein,

[1] L. Michaelis and H. Pechstein, *Biochem. Zeitschr.* **47**, 260. 1912.

it will be zero; at higher values of h it will be positive in sign, and at lower values negative. The surface tension at the boundary between the phases is reduced by the electric charge; and will therefore possess a maximum value when the charge is zero, i.e. at the iso-electric point. The greater the surface tension the greater is the tendency to precipitation.

Both explanations come to the same thing. For in the first case the question is left open as to whether the ions that go into solution are actually single ions; there might also exist aggregates of several single ions; it is necessary merely to assume that casein ions in general shew a higher degree of dispersion than iso-electric ("insoluble") casein, for increase in the degree of dispersion constitutes an approach to the dissolved condition, while a diminution of the degree of dispersion is a step towards the precipitated condition.

The second explanation appeals to the mind inasmuch as it offers in terms of surface tension an explanation of the reason why iso-electric casein should have a greater tendency to separate out than has charged casein. But, again, the first explanation has the merit of bringing the phenomenon into line with the relationships found in solutions of non-colloidal ampholytes, although in each case offering no attempt at explaining how it comes about that iso-electric casein is decidedly, and an iso-electric amino-acid relatively, insoluble.

Casein is neither a completely reversible nor a completely irreversible colloid. Once the casein has been dissolved, small changes of state in very dilute solutions are fairly readily reversible, and are determined unequivocally by the nature of the solvent. But so soon as it has been thrown down as a coarse precipitate from a stronger solution its re-dispersion by removal of the precipitating agent is only to be achieved with difficulty. The experiment must therefore be carried out in such a way that a coarse precipitation does not occur during the mixing of the reagents, except where this represents the final definitive condition. This condition is fulfilled in the following scheme:—

·2 gm. of fat-free casein (Hammarsten) is added to 5 c.c. N-sodium acetate solution (13·6 gm. of the crystalline salt [$CH_3 \cdot COONa + 3H_2O$] in 100 c.c. water). By the further addition of a certain amount of water and gentle warming the protein forms a faintly opalescent solution. This is diluted to 50 c.c. with

distilled water so that a solution of casein in ·1N. sodium acetate solution is obtained. Nine tubes are now made up as follows:

No.	1	2	3	4	5	6	7	8	9
Distilled water c.c.	8·38	7·75	8·75	8·5	8	7	5	1	7·4
N/100 acetic acid c.c.	·62	1·25							
N/10 acetic acid c.c.	—	—	·25	·5	1	2	4	8	
N/1 acetic acid c.c.	—	—	—	—	—	—	—	—	1·6

Finally to each tube is added 1 c.c. of the above solution of casein-sodium acetate solution; this latter addition is made as rapidly as possible by blowing in the casein solution from a 1 c.c. pipette, and at once giving a thorough shaking. An immediate turbidity is produced in some of the tubes according to the following scheme, in which the degree of turbidity in each case is denoted by the number of crosses.

No.	1	2	3	4	5	6	7	8	9
pH . . .	5·9	5·6	5·3	5·0	4·7	4·4	4·1	3·8	3·5
Turbidity . .	0	0	+	++	+++	++	+	+	0

After 5 minutes precipitation will have occurred in some of these turbid solutions, as follows:

No.	1	2	3	4	5	6	7	8	9
	0	0	+	+++	×××	××	++	+	0

The degree of precipitation is denoted by the diagonal crosses, so that the precipitation optimum occurs in tube 5 (pH=4·7).

The experiment is now repeated with the modification that 1 c.c. of the distilled water added to each tube is replaced throughout each series by 1 c.c. of one of the following solutions:

M. NaCl
M. $CaCl_2$
M/100 $ZnCl_2$
M. NaCNS
M/1000 $AlCl_3$

NaCl shifts the optimum a little and NaCNS more markedly to the right; so also to a certain extent does $CaCl_2$. $AlCl_3$ shifts the optimum to the left and at the same time inhibits the precipitation; $ZnCl_2$ shifts the optimum to the left without preventing the precipitation.[1]

[1] L. Michaelis and A. V. Szent-Györgyi, *Biochem. Zeitschr.* **103**, 178. 1920.

The way in which these various ions bring about a shifting of the precipitation optimum may be pictured as follows: in the presence of a certain concentration of hydrogen-ions the colloidal particles are completely discharged as a result of the combination of the positively charged hydrogen-ions with the negatively charged protein ions. But if there are also present in the solution other cations with which the protein ions are capable of combining to an appreciable extent not only will a smaller concentration of hydrogen-ions be required to bring about complete discharge of the particles, but the same concentration of hydrogen-ions will now suffice to produce a reversal of the charge. The greater the extent to which a given ion is taken up (" adsorbed ") by a protein the greater the shift that that particular ion will produce in the precipitation optimum.

EXERCISE 25.

Preparation of Denaturated Colloidal Serum Albumin and Determination of its Precipitation Optimum.[1]

Native serum albumin is a hydrophil colloid. Its degree of dispersion is so great that even at the iso-electric point it is not precipitated. When heated it becomes " denaturated," that is, it acquires the properties of a suspensoid: it is precipitated at the iso-electric point like casein. In fact its precipitation optimum occurs at its iso-electric point, but this differs from that of the native albumin. If albumin is heated in a solution whose h is sufficiently far removed from the iso-electric point and which contains the minimum of electrolytes it does not coagulate but forms a turbid milky suspension. Albumin solutions which have been purified by dialysis usually possess such a value of h that when sufficiently diluted they do not coagulate on heating, for they react almost neutral (pH about 7) while the iso-electric point of de-naturated albumin is at a pH of about 5·4. The traces of alkali which remain in the serum if it is not dialysed too thoroughly favour the stability of the dialysed albumin to heat. But these possible traces of alkali do not disturb the results of the further experiment as the behaviour of the protein is studied in well-buffered solutions. The conditions of reversibility of these changes are the same as hold for casein.

[1] L. Michaelis and P. Rona, *Biochem. Zeitschr.* **27**, 38. 1910.

7 c.c. of blood serum with a little added toluene are placed in a diffusion thimble (Schleicher and Schull's small form) and dialysed against frequent changes of distilled water for at least four days. Two or three such thimbles should be set up simultaneously. At the end of this time 5 c.c. of the fluid are sucked off without disturbing the precipitated globulin, diluted with 40 c.c. of distilled water and heated in a bath of boiling water. In a short time the liquid becomes turbid but does not precipitate, and after a further interval of 3—4 minutes the denaturation is complete. The solution will keep a long time without precipitating.

(a) Determination of the optimum value of h for precipitation in the presence of a constant minimum concentration of electrolytes.

The following series of tubes is made up:

No.	1	2	3	4	5	6	7	8
·1 N. Sodium acetate c.c.	1	1	1	1	1	1	1	1
·01 N. acetic acid c.c.	·1	·2	·4	·8	1·6	3·2	—	—
·1 N. acetic acid c.c.	—	—	—	—	—	—	·64	1·28
Distilled water c.c.	6·9	6·8	6·6	6·2	5·4	3·8	6·36	5·72
Test fluid c.c.	1	1	1	1	1	1	1	1
pH	6·7	6·4	6·1	5·8	5·3	5·0	4·7	4·4

"Test fluid" is the solution whose particular effect is to be studied. In this first experiment no such special solution is to be tested so that distilled water is used instead.

After the solutions have been well mixed, 1 c.c. of the above described solution of denaturated albumin is added to each with further shaking.

The degrees of turbidity and of precipitation are now observed. The experiment goes much more slowly than that with casein. The optimum solution shews a well-marked turbidity after a few minutes, but usually it is only after half an hour that the flakes are large enough to shew any obvious signs of settling. It is usually in tube No. 5 that the turbidity and precipitation occur first. The value of h for the contents of this tube is calculated as in a previous exercise. Gradual precipitation is also observed in tube No. 4 but in the immediate neighbours of these tubes only the merest suggestion of a precipitate can be made out. The optimum reaction for precipitation is the iso-electric point of the denaturated albumin: in the tube to the left of this (at a less acid

reaction) it carries a negative charge, and to right (in the more acid solution) a positive charge.

(b) In a second series, otherwise similar, N/3 NaCl is used as "test fluid." The precipitation is strongly inhibited: the position of the optimum is unchanged, but it can only just be recognised as the protein hardly separates out.

(c) M/6 Na_2SO_4 has an even more powerful inhibitory action.

(d) On the other hand 1M. NaI and 1M. NaCNS show no inhibitory action, but shift the optimum to the right, that is to the more acid side.

(e) $CuSO_4$, even in ·1 M. solution, and in some cases occasionally in ·01 M. solution, shifts the region of precipitation right over to the left end of the series.

(f) Acid dyes (Eosin serves well, but diamine-red is better, in ·1 per cent. solutions) shift the region of precipitation well over to the right, and the precipitate carries down the colour with it. (In the case of ·1 per cent. eosin the optimum occurs in tube No. 7.) With eosin even at the optimum only a turbidity is to be observed, but with diamine-red extensive precipitation occurs.

(g) Quinine and basic dyes (Trypoflavine in ·1 per cent. solution is recommended as its colour is not deep enough to produce serious difficulties of observation) shift the region of precipitation to the left and in the case of trypoflavine the precipitate is coloured. The optimum occurs in the tube on the extreme left of the series, and after an hour an extensive precipitation has occurred in tubes 1 to about 3.

The precipitation of denaturated albumin in the neighbourhood of the iso-electric value of h is therefore modified as follows:

1. The precipitation is inhibited in to an increasing degree throughout the series of anions:

Acid dyes, CNS, I, Br, Cl, SO_4 as alkali salts. (The first members of this series even facilitate the precipitation.)

This series of anions only holds when the salts are present in relatively small concentration (up to 1 M.) At greater concentrations the series is reversed: SO_4 which in dilute solution shews the greatest inhibitory effect now becomes the most powerful precipitant.

Among the cations only the trivalent ions shew a well-marked inhibitory action: the divalent cations facilitate the precipitation.

2. The optimum value of h for the precipitation is changed:

(a) towards the more acid side by the anions (in the form of alkali salts) in the following order of increasing effectiveness:

SO_4, Cl, Br, I, CNS, acid dyes.

(b) towards the less acid side by the cations (in the form of chlorides or sulphates) in the following order of increasing effectiveness:

Alkali cations, $Mg^{\cdot\cdot}$, $Zn^{\cdot\cdot}$, $Cu^{\cdot\cdot}$, $Al^{\cdot\cdot\cdot}$, basic dyes.

The action of these salts depends among other things on the circumstance that their ions combine to a certain extent with the protein. In this way they either co-operate or compete with the hydrogen ions in influencing the charge on the colloidal particles. The combination of the protein with the ions in question may be regarded as a process of salt-formation or of ionic adsorption, according to the point of view that one adopts with regard to the explanation offered on page 63. Whichever idea proves most acceptable, the fundamental explanation of the whole process of precipitation remains the same.

EXERCISE 26.

(*a*) The Sensitiveness of Gelatin to Alcohol at various Hydrogen Ion Concentrations.[1]

It is only certain proteins such as casein and the globulins that are precipitated when the reaction of their solutions is brought to the iso-electric value. Other proteins such as albumins, hæmoglobin and gelatin still remain in solution at this point. But even these can be shown to possess at the iso-electric point a maximum sensitiveness to agents that tend to produce precipitation without disturbing the balance of hydrogen or other ions in the solution. As an example we will shew that gelatin is most readily precipitated by alcohol at the iso-electric point.

[1] Quoted from Wo. Pauli, *Kolloidchemie der Eiweisskörper*, vol. 1, Dresden and Leipzig, 1920, p. 32. English translation by Thorne. London: Churchill, 1922.

The following solutions are made up:

No.	1	2	3	4	5	6	7	8	9
N/10 sodium acetate	2	2	2	2	2	2	2	2	2
N/10 acetic acid .	·12	·25	·5	1	2	4	—	—	—
N/1 acetic acid .	—	—	—	—	—	—	·8	1·6	3·2
Distilled water .	3·88	3·75	3·5	3	2	0	3·2	2·4	·8
1% gelatin sol. .	2	2	2	2	2	2	2	2	2

After shaking, 90 per cent. alcohol is added to tube 5 until a *just* visible cloudiness is slowly produced. In general some 8 c.c. of alcohol will be required. An equal volume of alcohol is then added to each of the tubes. After 30 minutes the appearance of the tubes will be as follows:

No.	1	2	3	4	5	6	7	8	9
Turbidity . . .	—	—	—	++	+++	±	—	—	—
pH	6·0	5·6	5·3	5·0	4·7	4·4	4·1	3·8	3·5

The alcohol has thus produced the strongest precipitation in tube No. 5. As a matter of fact it is known from cataphoresis experiments that the iso-electric point of gelatin occurs at a pH of 4·7.

After 24 hours the turbidities will have given place to precipitates. The optimum is now no longer so sharply defined; all the tubes to the left of the optimum shew a more marked degree of precipitation than those to the right. In this respect the solutions group themselves asymmetrically about the optimum.

(*b*) The Sensitiveness of Native Serum Albumin to Alcohol at various Hydrogen Ion Concentrations.[1]

10 c.c. of blood serum are treated with 10 c.c. of saturated ammonium sulphate solution. The mixture is filtered after half an hour and the filtrate is dialysed in a Schleicher and Schull thimble (see p. 67) against frequent changes of distilled water until no appreciable amount of sulphate can be detected in the outer fluid. This is partially achieved in five days, but a more complete removal of the sulphate requires 2 to 3 weeks. For our purpose the time of dialysis may be cut down to a few days if necessary. A little toluene should be added to the protein during

[1] Quoted from W. Pauli, l.c.

the process. In this way a solution of serum albumin free from globulin is obtained. The solution is diluted with an equal volume of distilled water before use with the following series:

No.	1	2	3	4	5	6	7	8	9
N/10 sodium acetate	1	1	1	1	1	1	1	1	1
Distilled water .	7·38	6·75	7·75	7·5	7	6	4	0	6·4
N/100 acetic acid .	·62	1·25	—	—	—	—	—	—	—
N/10 acetic acid .	—	—	·25	·5	1	2	4	8	—
N/1 acetic acid .	—	—	—	—	—	—	—	—	1·6

1 c.c. of the albumin solution is now well mixed with the contents of each tube. The solution in tube No. 5 is the one whose reaction corresponds to the iso-electric point of albumin (pH=4·7; but no precipitation occurs because albumin is one of those proteins whose degree of dispersion even at the iso-electric point is very high. Now if to each tube we add 6 c.c. of approximately 90 per cent. alcohol turbidities appear after a time according to the following scheme:

No.	1	2	3	4	5	6	7	8	9
pH	6·0	5·7	5·4	5·1	4·7	4·4	4·1	3·8	3·5
Turbidity after 10 minutes .	0	0	±	±	++	+++	±	0	0

The optimum reaction for the precipitation is thus very close to the iso-electric point. The small divergence is readily explained by the change produced in the dielectric constant of the solution by the 30 per cent. or so of alcohol that it contains, and the consequent change in the ionisation constants of the acetic acid, the water and the protein.

EXERCISE 27.

The Precipitation Optimum of a Mixture of Tannin and Gelatin.[1]

Two colloids which are present together in the same solution may mutually precipitate each other, even if they are hydrophil colloids. It is not usually possible to foretell in any given case how great will be this tendency to mutual precipitation, just as

[1] L. Michaelis and H. Davidsohn, *Biochem. Zeitschr.* **54**, 323. 1913.

it is generally impossible to say whether the salt formed by a given acid and a given base will be easily or sparingly soluble (for example the salt formed from acetic acid and caustic soda remains in solution while that obtained by adding phosphoric acid to baryta is precipitated). But it is possible to foresee what factors will influence this tendency to mutual precipitation in the case of a given mixture of colloids. For example, two amphoteric colloids of different iso-electric points will precipitate each other most powerfully in a solution whose reaction is such that the one colloid possess a distinct positive and the other a distinct negative charge. The sharpness of the optimum will depend on the affinity between the two colloids. A protein is precipitated by its specific precipitin with about equal readiness in faintly acid, neutral and faintly alkaline solutions (as for example, is $Ba(OH)_2$ by H_2SO_4) but a mixture of tannin and gelatin shews a fairly sharp optimum over a narrow range of reaction, just as does a mixture of $ZnCl_2$ and NH_4Cl, which will give a precipitate of $Zn(OH)_2$ only within a narrow range on the faintly alkaline range side when its reaction is changed by the addition of NaOH or HCl. The following series is set up:

No.	1	2	3	4	5	6	7	8	9	10
·5% gelatin solution c.c.	1	1	1	1	1	1	1	1	1	1
1 N.NaOH c.c.	1	1	1	1	1	1	1	1	1	1
Distilled water c.c.	6·5	6·4	6·3	6·1	5·7	4·9	3·3	·1	6·2	4·9
1 N. acetic acid c.c.	1·0	1·1	1·2	1·4	1·8	2·6	4·2	7·4	0	0
10 N. acetic acid c.c.	0	0	0	0	0	0	0	0	1·3	2·6

After thorough mixing 1 c.c. of ·1 per cent. tannin solution is added to each tube and the solutions are then immediately shaken again.

The result obtained within one minute of setting up the experiment is as follows:

No.	1	2	3	4	5	6	7	8	9	10
pH	>6	5·8	5·4	5·1	4·8	4·5	4·3	4·0	3·7	3·4
Turbidity after 1 min.	0	+	+	++	++	++++	+++	+	±	0

The relative depths of turbidity in the various solutions are represented by the crosses. The turbidity soon resolves itself into a precipitate. The sharpness of the optimum and its exact position depend on the relative quantities of gelatin and tannin present: the proportions given above are by far the most suitable: they give a quite definite optimum.

The values of h are obtained as follows: from the known quantities of acetic acid and caustic soda the molecular concentrations of free acetic acid and of sodium acetate are calculated. For example, tube 5 contains 1 c.c. of 1 N.NaOH and 1·8 c.c. of 1 N. acetic acid, so that the ratio of free acetic acid to sodium acetate is ·8 : 1.

Now $h = 2 \times 10^{-5} \times \frac{\text{acetic acid}}{\text{sodium acetate}}$, so that for the tube in question $h = 2 \times 10^{-5} \times \frac{\cdot 8}{1} = 1 \cdot 6 \times 10^{-5}$. Tube No. 1 contains only sodium acetate, so that its value of h is much smaller than that of No. 2 (2×10^{-6}) and not far removed from the neutral value (10^{-7}).

From this experiment it will be realised that when a colloidal solution is found to possess a precipitation optimum at a particular value of h it does not necessarily follow that there is only one colloid present and that this is being precipitated at its iso-electric point; there may be a mixture of colloids present and the optimum value of h for the precipitation may be simply that which is most favourable to their mutal interaction.

EXERCISE 28.

The Optimum Reaction for the Precipitation of Lecithin.[1]

About ·5 gm. of Merck's lecithin are agitated with 50 c.c. of distilled water in a mechanical shaker until all the lecithin has gone into a uniform cloudy emulsion.

A normal solution of lactic acid is prepared according to the method ordinarily adopted in analytical chemistry. The solution must be boiled for a quarter of an hour before its final titration in order to convert the lactone which is invariably present into lactic acid. The titration itself is made with 1 N.NaOH using phenolphthalein as indicator.

A N/40 solution of sodium lactate is now prepared by taking 5 c.c. of 1 N.NaOH, adding a drop of phenolphthalein, titrating with the lactic acid until the indicator is just decolourised and finally diluting the resulting solution to 200 c.c. with distilled water.

From further portions of the standard lactic acid solutions of ·1 molar and ·01 molar strength are prepared.

[1] Feinschmidt, *Biochem. Zeitschr.* **38**, 244. 1912.

The following series is now made up:—

No.	1	2	3	4	5	6	7	8	9	10	11	12
M/40 sodium acetate c.c.	1	1	1	1	1	1	1	1	1	1	1	1
·01 M. lactic acid	·49	·98	1·95	3·9	7·8	—	—	—	—	—	—	—
·1 M. lactic acid	—	—	—	—	—	1·56	3·12	6·28	—	—	—	—
1 M. lactic acid	—	—	—	—	—	—	—	—	1·25	2·5	5	10
Distilled water	9·51	9·02	8·05	6·1	2·2	7·44	6·88	3·75	8·75	7·5	5·0	0
Lecithin emul'n	1	1	1	1	1	1	1	1	1	1	1	1
pH	4·9	4·6	4·2	3·9	3·6	3·3	2·9	2·6	2·3	2	about 1·7	about 1·4
Result: (+turbidity; × precipitation).												
After 1 minute	—	—	—	—	—	—	+	—	—	—	—	—
,, 10 minutes	—	—	—	—	—	+	×	—	—	—	—	—
,, 30 ,,	—	—	—	—	—	+	×××	—	—	—	—	—
,, 60 ,,	—	—	—	—	—	×	×××	—	—	—	—	—

The values of h are calculated from the approximate formula:

$$h = 1{\cdot}5 \times 10^{-4} \frac{\text{(lactic acid)}}{\text{(sodium lactate)}}$$

so that for the optimum solution, No. 7, h is about $1{\cdot}2 \times 10^{-3}$; pH, 2·9.

Different optima are found for different preparations of lecithin. The optimum reaction is often found to be at the extreme acid end of this series and in many cases (alcoholic extract of heart muscle, for example) even to be outside this range altogether.

EXERCISE 29.

The Precipitation Optimum for the Lecithin-Protein Complex.

This experiment is carried out in exactly the same way as the previous one, except that a mixture of 20 c.c. of the lecithin emulsion +1 c.c. of dialysed blood serum is used in place of the lecithin emulsion alone.

Result: after one minute:

No.	1	2	3	4	5	6	7	8	9	10	11	12
	×××	×××	×××	++	++	++	0	0	0	0	0	0

In the tubes labelled 0 no intensification of the original turbidity due to the lecithin can be observed.

The region of precipitation is now shifted well over to the less acid end of the series, and at the same time the precipitate is much more bulky. For further remarks see exercise 27.

By means of a similar series, using blood serum diluted 20 times

with distilled water instead of with the lecithin sol it can be demonstrated that the formation of these massive precipitates cannot be brought about by the serum alone.

As sources of lecithin for this exercise extracts of heart muscle, Wassermann extracts, can be used.

The fact mentioned in the preceding exercise that lecithins from different sources possess different precipitation optima can be partly explained by the presence in these several preparations of variable traces of proteins of different kinds.

EXERCISE 30.

The Agglutination of Typhus Bacilli by Acids.[1]

Two sloping agar tubes are innoculated with typhus bacilli, and the innoculating material is spread over the entire surface of the agar by means of a drop of sterile water. After 24 hours' growth in the incubator the whole bacterial colony is washed off with 5 c.c. of distilled water (not sodium chloride solution), a second equal portion of distilled water is used to complete the washing off and the bacterial suspension is diluted to a volume of 20–30 c.c. A very distinctly turbid preparation should result. This is placed in a 20 c.c. burette, graduated in c.c. and provided with a glass tap. In order to avoid working with living pathogenic bacteria the suspension may be treated with one-tenth of its volume of 5 per cent. phenol solution and allowed to stand for a few hours.

The following mixtures are now made up:

No.	1	2	3	4	5	6
1 N.NaOH c.c. . .	5	5	5	5	5	5
1 N.acetic acid c.c. .	7·5	10	15	25	45	85
Water c.c. . . .	87·5	85	80	70	50	10

These solutions will keep unchanged for some time.

Their acidities are roughly:

h . . .	1	2	4	8	16	32×10^{-5}
pH . .	5·0	4·7	4·4	4·1	3·8	3·5

1 c.c. of each of these solutions is placed in a separate tube to each of which 3 c.c. of the bacterial suspension is also added. The rack of tubes is now placed in the incubator until the first sign of precipitation is to be seen (10 minutes—1 hour). The

[1] L. Michaelis, *Deutsche med. Wochenschr.* 1912, No. 21.

tubes are then brought out into the room when it will be found that the agglutination rapidly becomes more distinct. The optimum effect occurs in tube No. 3 ($h = 4 \times 10^{-5}$). According to the agglutinating power of the particular strain of bacillus used the precipitate will be fine or bulky, and will be entirely confined to tube No. 3 or will extend to the neighbouring tube on one or both sides. The tubes must not be shaken during the observation as is usual in serum agglutinin tests. If, however, the bacteria happen to become shaken up the observation can easily be repeated if the tube is left to stand for a time.

The ease with which the bacteria become agglutinated (but not the position of the optimum in the series) depends on the strain of Typhus, on the density of the suspension and on the nature of the nutrient medium. Should it not be sufficiently striking Bacillus paratyphosus–B may be used instead. This will always give a good agglutination with acids, with an optimum in tubes 5–6.

VI.

Surface Tension.

The surface of a liquid shews a continuous tendency to contract so that its area is a minimum. This is explained as being due to a tension that resides in the surface. It is on account of this tension that a fluid flows from a small aperture in a succession of drops; each drop separating only when its weight has become sufficient to overcome the supporting surface tension. This tension is also responsible for the fact that a liquid will rise in a capillary tube whose walls it wets. The condition of "wetting" would be satisfied if an extremely thin film of the liquid were to creep up the walls of the tube; but this would involve the production of a very large surface of fluid and this is prevented by the rise of the fluid in the tube.

The surface tension of water is changed by the presence of dissolved substances; there are not many such which produce any marked rise of surface tension, but many are known which produce a very considerable diminution of the magnitude of this property. These are known as capillary-active or surface-active substances. The most potent of them are compounds rich in carbon, and the longer the carbon chain and the smaller the number of decidedly electropositive and (particularly) electronegative groups (such as -OH and -COOH) the more powerful is their action. Surface active substances are distinguished by a high degree of adsorbability and by powerful biological actions such as inhibition of respiration, narcosis, etc.).

EXERCISE 31.

The Capillary-Tube Method.

A fluid which wets the tube will rise in it to a height which is given by the formula $h = \frac{2\sigma}{rD}$ where h is the height, σ the surface tension, r the radius of the capillary and D the specific gravity of the fluid. The height h is the difference of level between the level of the outer fluid and that in the capillary. But the determination of the level of the outer fluid requires special

apparatus. This, however, can be dispensed with by the use of two tubes as follows: suppose that the radii of the tubes are r_1 and r_2 respectively, then

$$h_1 = \frac{2\sigma}{r_1 D}, \text{ and } h_2 = \frac{2\sigma}{r_2 D},$$

$$\text{so that } \quad h_1 - h_2 = \frac{2\sigma}{D} \cdot \frac{1}{(r_1 - r_2)}.$$

This difference of height, $h_1 - h_2$ can be taken without appreciable error as being equal to the difference in level of the two menisci. If the same two tubes of different diameters are used throughout any one series of experiments we may write:

$$h_1 - h_2 = \frac{K\sigma}{D}, \text{ or } \sigma = \frac{(h_1 - h_2)}{K} \cdot D,$$

where K is a constant, whose value can be seen from the previous formula to be $\frac{2}{r_1 - r_2}$.

This constant can be evaluated for any pair of capillaries by observing the difference in the height to water will rise in them. If we desire only relative values of surface tension compared with that of water taken as unity then σ for water is $= 1$, D for water also $= 1$ so that $K = (h_1 - h_2)$ for water. The relative surface tension of a fluid is therefore equal to the difference of level to which it rises in two capillaries divided by the difference in level which is observed when pure water at the same temperature is used in the same capillaries, and multiplied by the specific gravity of the fluid.

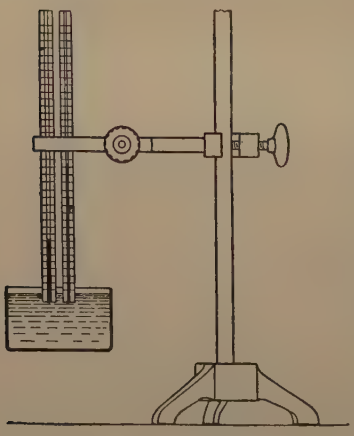

FIG. 8.—Double Capillary.

The measurement is made most conveniently by means of two lengths of thermometer tubing on each of which a scale of millimeters has been etched. (Fig. 8). In order to avoid errors of reading due to parallax the scale divisions should be carried at least halfway round the circumference of the tubes. The

outer diameters of the tubes should be approximately equal so that they they can be easily clamped together. The internal diameters may be conveniently 2·5 mm.; an ·34—·4 mm. respectively. Before use the capillaries must be cleaned in sulphuric acid-bichromate mixture; they are then supported as in fig. 8 and dipped into the fluid under investigation. It is necessary to be certain that the menisci move freely in the tubes, that no drops of liquid adhere to the internal walls of the tubes above the menisci, and that the fluid returns to its original levels after being displaced either up or down the tubes.

We may quote as an example the observation that at 18°C. water gave a difference of level of 36·5 mm. while a saturated solution of fermentation amyl alcohol gave a difference of 13·4 mm. Since the density of the solution was not appreciably different from that of water, its relative surface tension may be taken as equal to $\frac{13{\cdot}4}{36{\cdot}5} = {\cdot}367$.

EXERCISE 32.

Estimation of Relative Surface Tension by Counting Drops. (J. Traube's Stalagmometer.)

The stalagmometer as originally devised by Traube consists of a glass tube of the form shewn in fig. 9. There are initial and final marks respectively above and below the pear-shaped bulb. The lower end is ground flat and is pierced by the capillary orifice.

FIG. 9. Traube's Stalagmometer 1/5 natural size.

1. Calibration of the stalagmometer. The stalagmometer is clamped vertically and a vessel for the collection of the out-flowing fluid is placed below. Water is sucked into the instrument until it stands above the upper mark and is then allowed to flow out. As soon as the level of the water has sunk to this mark the counting of the drops is begun and is continued until the liquid has reached the lower mark. The most suitable stalagmometers are those which contain about 80 drops. The observation is repeated at least three times and consecutive countings should not differ by more than one drop.

Instruments which contain a smaller number of drops (for example 18 drops) are provided with extra graduations above and below both the upper and the lower marks. The total range covered by these extra graduations corresponds to the volume of half a drop above and half a drop below each of the main marks, so that if there are five divisions above and five divisions below each main mark each sale division will correspond to one-tenth of a drop. Since the falling of a drop will not in general exactly coincide with the passage of the meniscus through the upper main mark, the counting is started from the time when a suitable drop falls, the position of the meniscus at this instant being read off from the scale. The same procedure is adopted at the end of the counting, the position of the meniscus when the last drop falls being read off from the lower scale. Fractions of a drop above the initial mark must be substracted and fractions below must be added to the main number of drops counted, similarly fractions of a drop above the final mark must be added and fractions below substracted in order to arrive at the true number of drops included between the two main marks.

For example:

Nineteen drops were counted between the third scale division above the upper mark and the fourth scale division above the lower mark.

The true number of drops included between the two main marks was therefore $19 - \cdot 3 + \cdot 4 = 19 \cdot 1$ drops.

2. In making obeservations on an unknown fluid, several portions of it are first sucked through the capillary (as in the capillary-tube method) and then the drops are counted in the way just described for water. If N_w is the number counted in the case of water, and N that for the unknown liquid, and if D is the specific gravity of the latter and σ its relative surface tension, then

$$\sigma = \frac{N_w}{N} \cdot D.$$

In order to demonstrate Traube's series, that is the rapid increase of surface activity in homologous series as the length of

the carbon chain increases the surface tensions of the following solutions should be determined:

	relative surface tension
A. (a) 1 N. methyl alcohol (3·2 per cent. by weight or 3·9 per cent. by volume)	·92
(b) 1 N. ethyl alcohol (4·6 per cent. by weight or 5·75 per cent. by volume)	·76
B. (a) ·125 N. ethyl alcohol (·57 per cent. by weight or ·71 per cent. by volume)	·95
(b) ·125 N. (iso–)amyl alcohol (1·1 per cent. by weight or 1·36 per cent. by volume) ..	·54
C. A saturated solution of either heptyl or octyl alcohol (this will contain an amount of the alcohol almost too small to be detected analytically) about	·5

From the result of experiment A it is seen that in equimolecular solutions the higher alcohol is the more active. The same thing is seen in B from which it also appears that not only in equimolecular but also in the same absolute concentrations amyl alcohol is much more active than either methyl or ethyl alcohol. And lastly, in C it is shewn that even traces of octyl alcohol too small to be recognised except by smell, produce a greater lowering of the surface tension than the amyl alcohol.

If a saturated solution of decyl alcohol is prepared by gentle shaking (vigorous shaking produces an opalescent colloidal solution) the clear filtered liquid will be found to be practically devoid of surface activity, because the diminution of solubility consequent upon the increase in length of the carbon chain has more than outweighed the increase of surface activity. If the mixture obtained by vigorous shaking be filtered the opalescent colloidal solution so obtained shews a marked reduction of surface tension. This behaviour should be carefully noted.

EXERCISE 33.

The Increase of Biological Activity of Surface-active Substances in Homologous Series.

The object of this experiment is to determine the minimal concentrations of various monohydric alcohols required to kill

Bacillus coli in about 15 minutes. Into a series of test-tubes are placed:

Methyl alcohol . . . . . c.c.	9·6	6·4	4·3	2·8	
Water . . . . . c.c.	·4	3·6	5·7	7·2	
in a second series:					
Ethyl alcohol . . . . . c.c.	7·5	5·0	3·3	2·2	
Water . . . . . c.c.	2·5	5·0	6·7	7·8	
in a further series:					
n-Propyl alcohol . . . . . c.c.	3·6	2·4	1·6	1·1	·7
Water . . . . . c.c.	6·4	7·6	8·4	8·9	9·3
in a further:					
Saturated aqueous solution of amyl alcohol, i.e. about 2·8% c.c.	10·0	6·7	4·4	3·0	
Water . . . . . c.c.	0	3·3	5·6	7·0	
and in a last:					
Saturated aqueous solution of heptyl alcohol . . . . . c.c.	10·0	6·7	4·4	3·0	
Water . . . . . c.c.	0	3·3	5·6	7·0	

Throughout each of these solutions two loops full of an agar culture of Bacillus coli is to be diffused. The culture is best rubbed up on the dry side of the tube and then washed into the liquid. The time at which each tube is innoculated is noted. Fifteen minutes later a loop full of fluid is taken from each tube and sown on an agar plate. One plate will serve for about eight sowings if it is divided up into suitable areas. For one hour after the sowing the plates are kept uncovered in the incubator with the sown surface downwards in order to allow the traces of alcohol which have been carried over to evaporate. The plates are then covered and left in the incubator at 37°C. for 24 hours. At the end of this time the sowings are examined in order to determine in which growth has taken place, and which have remained sterile. The result usually found is that the minimal lethal doses for the various alcohols are:

Methyl alcohol . . .	64	per cent. by volume
Ethyl alcohol . . .	50	,, ,, ,,
Propyl alcohol . . .	20	,, ,, ,,
Amyl alcohol	2·8	,, ,, ,, (the saturated solution).

Heptyl alcohol does not kill under these circumstances in spite of its high degree of surface activity: it is too insoluble.

Proceeding further along the homologous series to decyl alcohol we find that its surface activity is too small to be detected by the stalagmometer: this again is because the substance is so slightly soluble. A biological object that is much more sensitive than bacteria (and even than the stalagmometer itself) is furnished by Paramecium. This is always to be obtained by allowing water to stand in contact with hay for a few days. If a drop of a saturated solution of decyl alcohol is added to a culture of Paramecium the animals are killed instantly and their structure is profoundly deformed.

EXERCISE 34.

The Relative Quantitative Analysis of a Capillary-active Substance.

The problem is to determine the relative concentration of a given solution of tri-butyrin (the tri-butyric ester of glycerol) in terms of the saturation concentration of this substance which, at the same temperature, is taken as unity.

A few drops of tri-butyrin are shaken for 10 minutes with 100 c.c. of distilled water[1] in a closed flask, and then filtered.

The first and last portions of the filtrates are collected separately and are rejected. From the middle portion the following dilutions are prepared:

No.	1	2	3	4	5	6	7
Saturated tri-butyrin solution c.c.	10	8	6	4	2	1	0
Distilled water . . . . . c.c.	0	2	4	6	8	9	10

The drop-volume of each of these solutions is to be observed, but for this purpose the slowly-dripping stalagmometer of Traube is not absolutely essential; a more rapidly acting dropping-pipette such as that shewn in fig. 10 may be employed.[2]

It has a capacity of about 3 c.c. and its lower tube, whose walls should not be too thin, is slightly tapered. It is most conveniently cleaned by means of sulphuric acid-bichromate mixture. The

[1] Instead of distilled water it is recommended to use the phosphate mixture described in the next exercise, and to carry out the two exercises concurrently.

[2] P. Rona and L. Michaelis, *Biochem. Zeitschr.* **31**, 345. 1911.

drops as they form should creep a short distance up the walls of the tube before falling off. This should occur quite uniformly round the circumference of the tube, and if this should happen not to be the case it can be corrected by rolling the dry end of the tube between the dry thumb and index finger with a certain amount of pressure. The most suitable pipettes are those which give 80–90 drops with pure water. This means a rate of $1-1^1/_2$ drops a second. Since at this rate of flow the drops separate rather too soon on account of their kinetic energy the surface tension is not exactly inversely proportional to the number of drops as is the case with the true stalagmometer. For example, the number of drops of a partially saturated solution of heptyl alcohol relative to that of water was $\frac{24{\cdot}6}{18{\cdot}5} = 1{\cdot}32$ when measured with Traube's stalagmometer, while the ratio came to be $\frac{142}{101} = 1{\cdot}41$ when measured by means of the more rapid dropping pipette. But of course each particular value of this ratio corresponds quite definitely to its appropriate concentration of tri-butyrin, so that for the purposes of the chemical analyses required for this and similar exercises the simple dropping pipette is to be recommended particularly on account of the great saving of time which its use involves.

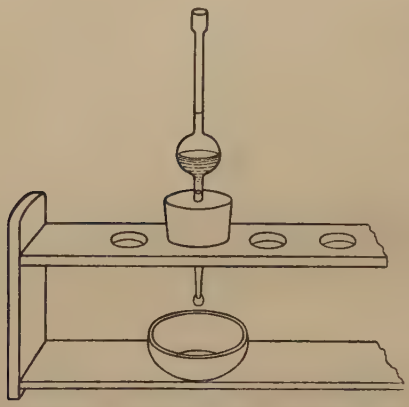

FIG. 10.—Dropping Pipette.

The drop numbers for each of the above seven solutions should be determined several times until the values obtained in parallel determinations do not differ by more than one drop. The agreement is usually not quite so good with the most concentrated solutions of tri-butyrin.

The following will serve as an example:

No.	1	2	3	4	5	6	7
Drop number (average values)	136·0	130·5	123·0	114·0	103·0	94·0	91·0

The values of concentration are now plotted as abscissae and the drop numbers as ordinates in order to obtain a calibration curve for tri-butyrin (fig. 11).

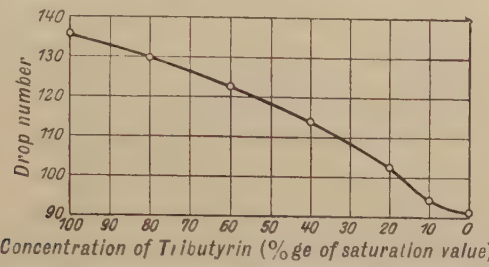

FIG. 11.—Calibration Curve for Tri-butyrin.

The concentration of any given solution of tri-butyrin can now be easily determined by measuring its drop number by means of the same pipette and reading off from the curve the concentration to which this value corresponds.

EXERCISE 35.

Demonstration of the Fat-splitting Ferment of Blood Serum.[1]

This exercise forms an immediate sequel to the preceding one, the calibration curve just obtained being applicable here also. But in this case the saturated solution of tri-butyrin is to be made up not in pure water but in a phosphate buffer, in order to produce and maintain the particular hydrogen-ion concentration that is most favourable for the action of the enzyme. The absolute concentrations of the phosphate buffer mixtures already described are smaller than is desirable for many purposes; we will therefore describe a method for the preparation of stronger buffer solutions. The starting point is a 1 molar (3N.) solution of phosphoric acid such as may be obtained of Kahlbaum. Its exact strength is determined in the following way: 10 c.c. of the acid solution are diluted with 100 c.c. of distilled water and then titrated with 1 N. NaOH using methyl orange as indicator. The titration is at an end when all traces of red tint have disappeared and the colour is the same as that of a strongly alkaline control solution obtained by adding a few drops of strong alkali and the same quantity of indicator (2–3 drops of a ·02 per cent. alcoholic solution) to an equal volume

[1] P. Rona and L. Michaelis, l.c.

of water. The last drop of alkali which removes the final trace of the orange tint and produces the pure pale yellow colour is not to be counted. Proceeding in this way, 10 c.c. of the acid should require exactly 10 c.c. of the 1 N. soda.

If phenolphthalein is used as indicator and the titration is made to include the last drop of alkali that first produces an undoubted rose tint, each 10 c.c. of the acid should require 20 c.c. of 1 N. soda.

Using this titrated acid, mixtures are made as follows:

1. 10 c.c. 1 M. phosphoric acid + 10 c.c. 1 N. NaOH + 10 c.c. of distilled water. This mixture is M/3 primary sodium phosphate.

2. 10 c.c. of 1 M. phosphoric acid + 20 c.c. of 1 N. NaOH: this is M/3. secondary sodium phosphate.

A mixture is now made of 1 c.c. M/3 primary sodium phosphate + 7 c.c. of M/3 secondary sodium phosphate + 100 c.c. of water. This solution is then shaken for 10 minutes with about 20 drops of tri-butyrin, and filtered, the first few c.c. of the filtrate being rejected and the remainder collected. To 50 c.c. of this solution 2 c.c. of human blood serum are added. This serum may be 2–3 days old, but must not have been inactivated by warming. A determination of the drop number of the mixture is made immediately after the addition, by the method described on p. 83, and this determination is repeated at intervals of 5–10 minutes. The values so obtained are plotted on a diagram in which the abscissae represent the times taken from the moment of addition of the serum and the ordinates the drop numbers. By the use of the calibration curve obtained in the last exercise the drop numbers may be converted into concentrations of tri-butyrin relative to the saturation concentration taken as unity, so that we may also plot concentrations of tri-butyrin against time. For example:

Time in minutes . . .	0	1	13	23	49	60
Drop number	147	147	138	134	125	123

Drop number for water = 83.

This method can be made to give a relative value for the amount of lipase present in the following way: the experiment is carried out with a number of specimens of normal human serum, and from the diagrams expressing the relation between time and drop number the various times at which the several solutions possess equal surface tensions are read off. It is not necessary to convert the readings into actual concentrations of tri-butyrin. For example,

suppose that a mixture made up with the serum replaced by an equal volume of water gives 140 drops. This is the true initial value. The first reading with the serum mixtures will already be slightly less than this. We now read off from the diagrams the times at which the drop numbers of the mixtures have fallen to (say) 120. For five different samples of normal serum these might be 10, 11, 12, 9, 9 minutes respectively, the average value being 10 minutes. Let us further note the times at which the drop numbers have fallen to 110: these might be 18, 19, 20, 16, 17 minutes respectively, with a mean value of 18 minutes.

These numbers can be used as a permanent standard of reference, provided that the temperature at which the subsequent determinations are made is the same within 2 or 3°C. Suppose, for example, that the pathological serum from a case of severe pulmonary tubercolosis gave

120 drops after 30·0 minutes
and 110 ,, ,, 58·0 ,,

we should conclude from the first of these readings that it takes 30 minutes for the same amount of decomposition to take place as occurs after 10 minutes with normal serum, while from the second reading we should infer that it takes 58 minutes to produce in the patient's serum the same change as would have occurred in 18 minutes in a normal specimen. Since the times required for equal amounts of decomposition are inversely proportional to the amounts of ferment present, the relative concentration of ferment in the pathological serum, referred to the normal concentration taken as unity, is $\frac{10}{30}$ from the first reading and $\frac{18}{55}$ from the second, i.e. ·33 and ·31 respectively, with a mean value of ·32.

EXERCISE 36.

Measurement of the Lipolytic Power of Gastric and Intestinal Juices.[1]

The principle is the same as in the case of the blood lipase, but the optimum value of h for the activity of these ferments is a different one.

To 90 c.c. of water 10 c.c. of the M/3 primary sodium phosphate

[1] H. Davidsohn, *Biochem. Zeitschr.* **45**, 284. 1912.

are added, and this solution, without the addition of any secondary phosphate, is saturated with tri-butyrin and filtered. To 30 c.c. of the filtrate 1 c.c. of the carefully filtered gastric juice is added, and the determination is carried out exactly as with blood serum.

In the case of the pancreatic lipase a solution of 90 c.c. of water + 10 c.c. of M/3 secondary phosphate is used, no primary phosphate being added. In such a solution the lipases of pancreatic and intestinal juices (and of blood) are active, but the gastric lipase shews at the most mere traces of activity. Conversely, in the solution recommended above for the gastric lipase, the pancreatic and intestinal lipases hardly work at all.

In order to determine whether the lipase present in a given sample of gastric contents is true gastric lipase or intestinal lipase that has been regurgitated through the pylorus, parallel experiments are made with both of the above phosphate solutions. Gastric lipase acts much more rapidly in the acid than in the more alkaline solution, while the intestinal enzyme acts more rapidly in the latter. If no appreciable difference of rate is noticed in the two media, a mixture of the two ferments is present in the gastric contents.

EXERCISE 37.

The influence of Capillary-active Substances on the Rate of Sedimentation.[1]

Into each of three test-tubes is placed 2 gm. of kaolin and 20 c.c. of water. No further addition is made to the first, while to the second a few granules of thymol and to the third a few particles of camphor are added. All three tubes are now shaken vigorously and then allowed to stand. The kaolin slowly separates out shewing a well defined boundary. After about an hour it will be found that the level of the kaolin has sunk about 10 mm. in the tube of pure water and by about twice the distance in the other tubes.

It should be pointed out (see the later section on Adsorption) that surface-active substances are not adsorbed by kaolin to any recognisable extent. In the case of charcoal, by which they are very well adsorbed, they exert no influence on the rate of sedimentation. The important biological effects produced by capillary-active substances even at surfaces at which they connot be proved to be adsorbed has not yet been explained.

[1] P. Rona and Györgyi, *Biochem. Zeitschr.* **105**, 133. 1920.

EXERCISE 38.

Conditioned Surface-Activity. Influence of Hydrogen-ion Concentration on Surface Tension.[1]

Electrolytes are known whose effect in reducing the surface tension of water is not a simple one but, other things being equal, depends on the prevailing value of h. This conditioned surface-activity may be associated with the cation of the electrolyte, as in the case of quinine, eucupin and many other alkaloids, or with the anion, as in the case of undecylic acid the commoner higher fatty acids and their alkali salts. We shall study the case of eucupin bi-hydrochloride. A 1 in 1000 solution of this substance will give 113 drops from a pipette that would give 84 drops when pure water is used. This solution is strongly acid on account of the hydrolysis of the salt. If now the acidity is diminished by means of phosphate buffers the drop number increases—at first gradually, and then with a jump to a maximum value.

The following solutions are made up:

No.	1	2	3	4	5	6	7
·1 N. NaOH c.c. . .	—	—	—	—	—	—	2·0
M/15 primary phosphate	2·0	1·4	·98	·69	·48	·34	—
M/15 secondary phosphate	0	·6	1·02	1·31	1·52	1·66	—
1% eucupin soln. . .	4·0	4·0	4·0	4·0	4·0	4·0	4·0
pH (approx.) . . .	5	6·3	6·8	6·9	7·1	8	12·6
Drop number . . .	124	176	187	187	185	169*	93†

(in pure solution = 113).

*At this degree of alkalinity a turbidity first appears. The drop number of the turbid solution was at first 213. The fluid was then filtered and the filtrate when examined several times during the course of an hour was found to give a constant drop number of 169.

† In this case an immediate precipitate is produced. The value given was obtained with the filtrate.

The drop number is thus a little higher in the pure primary phosphate solution (pH = about 5) than in a pure aqueous solution of eucupin bi-hydrochloride, which, in consequence of the hydrolysis of the salt is very acid. As the acidity diminishes the drop number shews a sudden increase at about pH 6 and attains a maximum value at about pH 6·8 (if one considers only the definitive values

[1] J. Traube and R. Somogyi, *Internat. Zeitschr. für physikochem. Biol.* 1 (1914); also W. Windisch and W. Dietrich, *Biochem. Zeitschr.* **97**, 135, 1919; **100**, 130, 1919, and other papers ibidem.

obtained with the filtrates) and then from pH 8 on begins to fall. At pH 12 the drop number has sunk almost to that found for pure water.

The phenomenon can be explained in the following way: The surface-active material is the free eucupin base (not the eucupin ion) and this is present in small concentration even in the aqueous solution of the salt on account of the hydrolysis. As the acidity diminishes, the concentration of the free base increases. Like all really powerfully surface-active substances it is but sparingly soluble in water and only shews a marked stalagmometric effect when it is present in supersaturated or finely dispersed colloidal solution (cf. the behaviour of decyl alcohol, p. 81). If the solution is made only faintly alkaline the liberated base remains in supersaturated (or colloidal) solution (tubes 2–5): at more alkaline reactions (tube 6) the base is to a great extent precipitated out—during the first few moments the solution shews a high drop number, but this diminishes in course of time: while at the highest degrees of alkalinity (tube 7) the base is simply quantitatively precipitated and the drop number sinks to that given by pure water.

As in other cases, the surface-activity of capillary-active substance is influenced by other ions in addition to $H^{\cdot}$ and OH'. The same ionic series are found here as in the case of other activities of ions. But here again the $H^{\cdot}$ and OH' ions are by far the most active. The substances that shew conditioned surface-activity belong to the classes that produce the most powerful pharmacological effects.

EXERCISE 39.

Titration with a Conditionally Surface-active Substance as Indicator.[1]

The property of changing their surface-activities suddenly at a given value of pH renders substances like eucupin suitable for use as indicators in acidimetric titrations. The method is particularly indicated for the titration of strongly coloured or of turbid solutions, in which the end point of a coloured indicator could not be observed. For purposes of demonstration we will titrate ·1 N. ammonia against ·1 N. HCl.

[1] Windisch and Dietrich, l.c.

To 10 c.c. of ·1 N.HCl in a porcelain dish 10 drops of a 1 per cent. solution of eucupin bi-hydrochloride are added. The mixture is titrated with the ·1 N. ammonia the drop number being determined after each addition by sucking up a suitable volume of the solution into the dropping pipette and allowing it to drop back into the dish. The following values are typical:

c.c. of ·1 N. ammonia added	Drop number
·0	92
5·0	92
7·0	92
9·0	95
9·5	97
10·0	125
10·5	122

Thus the sudden increase of drop number occurs after the addition of 10 c.c. which is the expected end point of the titration. This roughly corresponds with the end-point that would be given by methyl orange.

VII.

Diffusion, Osmosis, Filtration.

A substance occurring in dilute solution shews many points of resemblance in its behaviour in its solvent to that of a gas in an empty space. For example, the dissolved substance tends to distribute itself throughout the whole volume of the solvent, the driving force for this movement being known as diffusion. If the diffusion is prevented by means of a semi-permeable partition the dissolved substance exerts an osmotic pressure. We can therefore regard the osmotic pressure as the cause of the diffusion. Every particle which carries out its own individual movement independently of the Brownian movement of neighbouring particles contributes an equal share to the total osmotic pressure of the solution, no matter whether it be an ion, a single molecule or a gigantic complex of molecules such as a protein or a particle of mastic.

If the dissolved substance is a non-electrolyte the gas laws are directly applicable to it. In the case of electrolytes electrostatic forces come into play between the ions themselves and between these and the water molecules—forces which have to be taken into account in considering the case of free diffusion and which play a part of fundamental importance in determining the diffusion through a membrane. It is at once evident that the osmotic phenomena displayed by electrolytes will depend on the nature of the membrane employed.

A membrane that is permeable to the solvent, but not to substances in solution or in suspension, is termed semi-permeable. The most completely semi-permeable membranes are the precipitation membranes of M. Traube made in the form of a Pfeffer's cell (for example from potassium ferrocyanide and copper sulphate): they are completely impermeable to all simple salts. Parchment, collodion and bladder have larger pores, and the retention of a substance by these membranes is, by definition, an indication of the colloidal state.

If such membranes are used as filters, the pure solvent will pass through under the influence either of its own hydrostatic

pressure or of an external applied force, a process known as ultra-filtration, and here again the different membranes shew their particular permeabilities for molecules and complexes of various sizes.

EXERCISE 40.

Diffusion.

The rate of diffusion of a dissolved substance in solution is best demonstrated by allowing it to diffuse into solidified gelatin, the rate of movement under these circumstances being approximately, although not quite, identical with that which would be observed in pure water. Each of a series of tubes is filled to a depth of 10 cm. with a 10 per cent. solution of gelatin and when this has solidified the solutions to be tested are poured on top. Suitable examples are furnished by:

	Depth of diffusion after 24 hours
10 per cent. $CuSO_4$	10·0 mm.
1 in 1000 Eosin	5·0 „
1 in 1000 Methylene Blue	3·0 „
1 in 1000 Congo Red	0 „
Dilute hæmoglobin solution (laked blood)	·7 „
Gum mastic sol (see p. 12)	0 „
Soluble Prussian Blue	0 „

So far as substances in a state of molecular dispersion are concerned the diffusion rate decreases as the molecular weight becomes greater. Colloidal solutions diffuse much more slowly still, coarse suspensoids shewing hardly any movement at all.

It is to be noticed that hæmoglobin shews a well-marked rate of diffusion.

A very beautiful experiment is the preparation of Liesegang's rings. 4 gms. of gelatin are dissolved, with warming, in 120 c.c. of water and ·12 gm. of potassium bichromate are dissolved in the solution. The mixture is used to make quite thin films on the bottom of several Petri dishes and also to make columns about 10—15 cms. high in test-tubes. When the gelatin has set completely, a drop of 8·5 per cent. $AgNO_3$ solution is placed carefully in the centre of the film in a dish which is then covered and allowed to remain completely at rest. On top of each of the gelatin columns

in the tubes 5 c.c. of the same silver solution is placed very carefully by allowing it to run slowly from a pipette down the walls of the tube. Next day it will be found that in the dishes concentric rings and in the tubes horizontal layers of brown silver chromate have separated out, and the number of these is found to increase from day to day. The distances between the successive rings or layers becomes greater the further from the centre or the upper surface of the gelatin respectively they are formed. These results are to be explained as follows: The silver salt diffuses into the jelly and there forms the sparingly soluble silver chromate which at first remains in supersaturated solution. As soon as the degree of supersaturation has reached a certain value, as a result of the further diffusion of the silver nitrate, the silver chromate suddenly separates out so that the condition of supersaturation is removed along a certain line. The silver chromate in other still supersaturated regions now diffuses into this localised region of crystallisation and is there precipitated, until the supersaturation has completely disappeared. The silver nitrate now continues its outward diffusion until the same process is repeated. A necessary condition for the appearance of the phenomenon is that the precipitated silver nitrate should not form so thick a membrane that it is impermeable to further quantities of silver nitrate. A copper ferrocyanide membrane, for example, is impermeable to all salts, and so would block the way to all further diffusion as long as it possessed the mechanical strength required to stand the osmotic pressure.

EXERCISE 41.

Dialysis.

Dialysis is the process of separating colloidal from diffusible substances by taking advantage of the spontaneous osmosis of the latter through membranes.

Parchment sacs form the most permanent dialysing membranes, and of these the most useful are the excellent[1] diffusion thimbles of Schleicher and Schüll—particularly the small form holding

[1] It is much to be regretted that these thimbles, once so uniformly good, nowadays not uncommonly contain cracks that can only be detected during use.

about 10 c.c. About 5 c.c. of fluid should be used in each, and they should be placed in small beakers containing water to the same level. If ·85 per cent. NaCl solution is placed inside it is only a few minutes before chloride can be detected in the outside fluid, but if blood serum is used no protein can be detected in the outer fluid even after 24 hours. Eosin will be found to pass through readily from a 1 per cent. solution. At first the dye becomes firmly adsorbed on to the membrane, but later, when the adsorption equilibrium has been attained the diffusion begins. On the other hand Congo red will not pass through the sacs.

Of other dialysing sacs those of collodion are of first importance. They may be made as described later on pp. 97-98 or according to the following method due to Wo. Ostwald. A Schleicher and Schüll extraction-thimble made of filter-paper (not parchment) is filled with the collodion solution which is then at once poured out again. Then during the drying of the collodion film adhering to the walls the thimble is held in a horizontal position and rotated about its long axis. A plain cylinder can also be made by sticking the overlapping edges of the rolled filter-paper together by means of some of the collodion solution and then sticking on a bottom at one end of the tube in a similar way. The inner surface is then wetted with collodion solution as before, care being taken to reinforce the walls at the joins. After drying, the sacs should be washed in water, when they will remain for a long time ready for use, provided that they are kept permanently moist.

In the dialysis of blood serum with different membranes different end results are obtained particularly with regard to the amount of water drawn in by osmosis.

In a first experiment a small Schleicher and Schüll diffusion thimble made of parchment paper is used. After it has been well soaked in water 5 c.c. of blood serum are placed in it and it is then immersed in water in a small beaker (preferably a slightly conical one narrower at the top than at the bottom) in such a way that the levels of the liquid inside and out are approximately the same. In a parallel experiment ·85 per cent. NaCl solution is used as outer fluid instead of water. These outer fluids are changed several times at half-hourly intervals and then the apparatus is allowed to stand overnight. Next day the contents of each thimble are poured into a measuring cylinder when it will be observed that there has been hardly any increase of volume of the serum

that was dialysed against the NaCl solution, while that which was dialysed against the water has increased its volume by about one-third and it contains a precipitate of globulin.

A similar pair of parallel experiments is carried out, using a collodion sac made according to the directions on pp. 97-98. It can be made of the same diameter as the parchment thimble but preferably a little higher. In this case it will be found after 24 hours that the volume of the serum has increased considerably not only during the dialysis against water but also during that against the salt solution.

In neither of the four experiments will protein be found to have diffused into the outer fluid.

EXERCISE 42.

Compensation Dialysis.[1]

When a diffusible substance is present in a colloidal solution there exists the possibility that a part of it is bound to the colloidal particles, and the problem therefore arises of determining what proportion of it is so combined and what proportion remains free. These considerations apply, for example, to the determination of the concentration of free sugar or of free Ca-ions in serum. The problem can be solved by the method of compensation dialysis. The total sugar present in the serum is first determined on one specimen, and then further specimens are used for a series of parallel experiments in each of which as large a quantity as possible (about 50 c.c.) is dialysed against the minimum volume of one of a series of solutions of sugar of different known concentrations made up in ·85 per cent. NaCl solution. The object is to discover which of the sugar solutions neither increases nor diminishes in concentration during the dialysis. The concentration of this solution is equal to that of the original serum. As this particular problem involves a large number of chemical analyses we will give the details of another example[2] which is to a certain extent analogous. We will determine whether heptyl alcohol is adsorbed by colloidal ferric hydroxide.

25 c.c. of the colloidal ferric hydroxide (Liquor ferri oxychlorati

[1] L. Michaelis and P. Rona, *Biochem. Zeitschr.* **14,** 476. 1908.
[2] L. Michaelis and P. Rona, *Kolloidzeitschrift.* **25,** 225. 1919.

dialysati duplex, Merck) are placed in a small slightly conical beaker. 5 c.c. of a filtered saturated solution of heptyl alcohol are placed in a Schleicher and Schüll dialysing thimble, this is immersed in the colloidal iron solution and the beaker is then closed air-tight with a paraffined cork. A parallel experiment is set up using distilled water instead of the colloidal iron solution. After 2 or 3 days the contents of each thimble are investigated by means of the stalamometer. The result might be that in each case 116 drops would be given by a pipette that gives 85 drops of water. Now a solution of heptyl alcohol that gives 116 drops would give only 114 when diluted with 10 per cent. of its volume with water and 111 drops if diluted with to the extent of 20 per cent. We therefore conclude that the heptyl alcohol has not been adsorbed by the colloidal particles—at least not to the extent of 10 per cent.

EXERCISE 43.

Osmosis.

If a solution is separated from the pure solvent by a membrane that is permeable to the solvent itself but through which the solute will pass with difficulty or not at all, the solution inside draws water through the membrane. Completely semi-permeable membranes such as those of copper ferrocyanide are difficult to deal with. Collodion is much easier to work with, but can be regarded as semi-permeable only to a very limited degree. In the case of a completely semi-permeable membrane the solvent would be drawn in until the liquid stands at a certain height which measures the osmotic pressure of the solution. In the case of a membrane of incomplete semi-permeability such as collodion[1] no definite osmotic equilibrium is set up, because the dissolved substance diffuses out and finally equalises its concentration on both sides of the membrane so that the final result is that the liquid stands at the same level inside and out. But in this case the osmotic water current is easy to observe and the pressure of this current can be measured in spite of the change of its value with time.

A broad glass cylinder such as a wide measuring cylinder of

[1] Quoted from Lillie, *Amer. Journ. of Physiol.* **20,** 127. 1907; S. P. L. Sörensen, *Zeitschr. f. physiol. Chemie.* **106,** 1. 1919; Jacques Loeb, *Journ. of General Physiology.* 1 (several articles), 1918.

25 c.c. capacity is filled with collodion solution most of which is then poured out. The remainder is allowed to dry on the walls while the cylinder is held in a horizontal position and rotated continuously. A second layer of collodion is then poured in and allowed to dry in the same way as the first. After the collodion has set it is given a further period of drying. The collodion sac can then be carefully separated from the glass and drawn out of the cylinder. It is now washed for a time and then half filled with distilled water. The upper part is well dried and fitted with a bored rubber cork which carries a stout walled glass tube about 30 cm. long and of 1 mm. internal diameter. This should be furnished with a scale of millimeters and may conveniently be improvised from a 1 c.c. graduated pipette (fig. 12). The rubber cork must be cemented in air-tight by means of a ring of collodion, which is best applied before the sac has come into contact with water at all. When the collodion used to fix the cork in position is dry a few air bubbles are carefully blown down the glass tube. If all is air-tight the water will rise in the tube and will maintain its level. The tube is now clamped in a stand and the sac is arranged to dip into a vessel of distilled water. The level of the water in the tube now falls so slowly that it may be taken as constant over a considerable time; or the rate of fall may be noted and introduced as a correction into the results of later experiments.

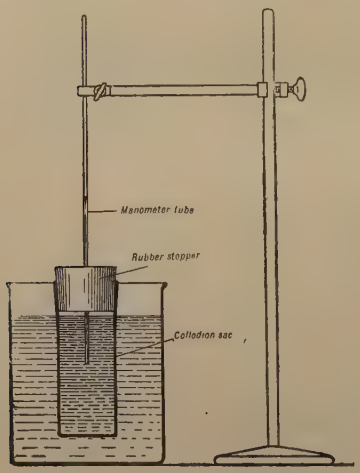

FIG. 12.—Simple Collodion Osmometer.

If one collodion sac is to be used for a long series of experiments it is necessary to be able to open the osmeter in order to change the fluids. This is done most simply by arranging so that while the glass tube passes air-tight through the rubber cork it is sufficiently loose to be withdrawn. A pipette can then be introduced through the hole in the cork for the purpose of sucking out the old fluid, washing out with water, and finally running in the new solution. Such an osmometer can be used over and over again. The most

important essential is the choice of a suitable brand of collodion; that which is not elastic cannot be used for this purpose.[1]

The collodion sac may be filled first with 10 per cent solution of cane sugar, and placed in distilled water. The liquid rapidly ascends in the glass tube. The experiment is then repeated with M/64 (·54 per cent.) cane sugar solution. In this case hardly any rise will take place owing to the great reduction of the value of the osmotic pressure.

When solutions of electrolytes are used the relationships are considerably modified and can no longer be explained in terms of the simple laws of osmotic pressure.

The rate of passage of the water is influenced very largely by the nature of the electrolyte itself and on certain points in the preliminary treatment of the collodion membrane. Let the collodion sac be soaked for 24 hours in a 1 per cent. aqueous solution of gelatin and afterwards wash it for some time in warm water. The properties of a membrane so prepared can be demonstrated by means of the following experiments.[2]

The sac is filled in turn with the following solutions which have equal osmotic pressures:

1. M/64 cane sugar
2. M/128 NaCl
3. M/192 $CaCl_2$.
4. M/192 Na_2SO_4

In spite of the equality of their osmotic pressures these solutions shew very different behaviour in the osmometer. For example, after 10 minutes the rise of the liquid in the tube might well be somewhat as follows:—

1. Cane sugar: hardly appreciable
2. NaCl: 11 mm.
3. $CaCl_2$: 22 mm. (after 40 minutes about 80 mm.)
4. Na_2SO_4: 3 mm.

Even negative osmosis is observed with some solutions—that is water passes from the solution into the pure water outside. Using a membrane of gelatinised collodion this phenomenon can

[1] Suitable collodion can be obtained from the Chem. Fabrik auf Aktien (formerly E. Schering) Berlin, N. It is best to order it in the form of solution "Kollodium D.A.B. V." and to dilute this with half its volume of a mixture of equal parts of absolute alcohol and ether.

[2] Jacques Loeb, l.c.

be observed with very dilute solutions of HCl or $AlCl_3$. If the sac is filled with pure water (the reverse of the usual procedure) and it is immersed in N/1000 HCl the fluid will have risen 5 mm. in the tube by the end of 10 minutes. With higher concentrations of HCl the osmosis is again positive.

As can be gathered from these last remarks on the high degree of activity shown by these very dilute solutions of HCl the magnitude and direction of the osmosis occurring at a gelatinised collodion membrane is very much influenced by the hydrogen-ion concentration of the solution. As a consequence of the variations in the value of h for the distilled water, and also possibly on account of the contamination of many samples of gelatine with some very active trivalent ion or other, experiments 2—4 described above sometimes show a feeble negative osmosis instead of the expected positive result. In this case it is necessary merely to add a trace of NaOH (at the most up to a concentration of N/300) to both inner and outer fluids in order to produce an energetic reversal of the sign of the osmosis.

EXERCISE 44.

Ultrafiltration.

A filtering membrane that is impermeable to colloids is termed an ultra-filter.

The simplest of ultra-filters can be made thus.[1] A filter "hat" of Schleicher and Schüll, or even an ordinary filter paper, is carefully fitted into a funnel and soaked thoroughly with warm water. When the water has drained off, the damp paper is soaked with collodion solution. This is then poured off as completely as possible and the remaining thin film is given a preliminary drying by rotating the funnel with its stem horizontal. The funnel is then placed stem downwards and left to dry for a further 10 minutes. A second thin layer of collodion is now applied and when excess of the solution has been poured off the funnel is allowed to dry again for 10 minutes, this time with the stem upwards. The filter is now rinsed for 10 minutes in water. Such a filter will allow water to pass through it under the influence of its weight alone, but of course more quickly under the action of a pump. As the filter does not fit air-tight to the funnel only a feeble vacuum can be

[1] Wo. Ostwald, *Kolloid-Zeitschr.* **22**, 143. 1918.

created by the pump—but this is quite sufficient. The density of the filter is now tested by means of mastic sol, prepared as directed on p. 12 and diluted until it is only faintly turbid. The permeability of the filter varies with the strength of the collodion solution used in its preparation. A filter made from the ordinary 4 per cent. collodion solution obtainable from the druggists will let through hardly a trace of protein from blood serum 10 times diluted with water. The permeability of the filter is further tested by means of Night-blue, Congo red and Collargol.[1] Dense filters will allow neither of these substances to pass, those of moderate density will keep back night-blue and Congo red while the most permeable of all will retain only night-blue. The quality of the collodion used makes a great difference to the permeability.

The impermeability of a filter to disperse particles and molecules is doubtless due in part to a lack of correspondence between the sizes of the particles and the dimensions of the pores of the filter. But this must not be regarded as the only factor which is operative. For example, mercury will not pass spontaneously through the pores of an ordinary filter paper or through a capillary glass tube whereas it will flow quite readily through a metallic capillary. The theory of ultrafiltration is still very incompletely developed.

This may be illustrated by the following simple facts: If a solution of Congo red is passed through one of these ultrafilters the filtrate is colourless; it seems as if the pores of the filter are too narrow to permit the passage of the molecules or micellae of the dye. Meanwhile the filter becomes stained red. Now let blood serum somewhat diluted with ·85 per cent. NaCl solution be filtered through the same filter. As a rule a trace of protein will come through, and this protein will carry the Congo red with it so that the filtrate is now rose-red in colour. Thus Congo red may be washed out of the filter by means of protein. The explanation of these phenomena seems to be as follows: the collodion membrane takes on a strong negative charge with respect to the aqueous solution and so also do the particles of Congo red, and as a result of the repulsion between these like charges the dye is prevented from passing the filter. When the pores have been coated with protein they may have become narrowed; but they cannot possibly have been increased in size. Their charge, however, will almost

[1] Wo. Ostwald, *Kolloid-Zeitschr.* **22**, 143. 1918.

completely have disappeared for the approximately neutral solution has a reaction not far removed from the iso-electric point of the protein. The electric repulsion of the colloidal particles will therefore be much weaker.

EXERCISE 45.

The Depression of the Freezing Point.

The osmotic pressure of true solutions is most commonly measured by means of the depression of the freezing point or the elevation of the boiling point, both of which are proportional to the osmotic pressure. We will give one example of the freezing point method, using a solution of cane sugar or of NaCl. The apparatus used for the purpose is that devised by Beckmann (fig. 13) which consists of a large outer vessel C fitted with a cover provided with three apertures. Of these the small side one carries a stirrer, the second carries a small thermometer (not shewn in the figure) while the large central aperture carries a cylindrical tube B provided with a bored cork. This supports a narrower cylindrical tube A which is provided with a side arm and forms the actual freezing vessel. Through its cork pass the Beckmann thermometer D and a stirrer.

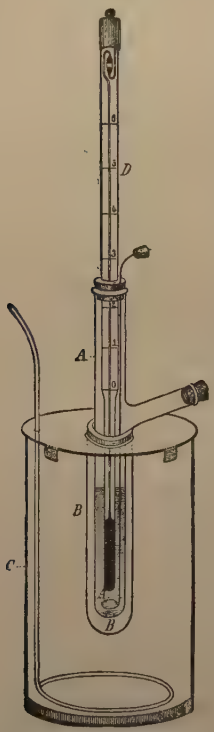

FIG. 13.—Apparatus for the Determination of the Freezing Point.

The Beckmann thermometer has a range of about 6°C. with scale divisions to hundredths of a degree so that thousandths can be estimated. It is not intended to give absolute readings of temperature; on the contrary the effective quantity of mercury can be changed by means of the mercury reservoir provided at the top of the instrument in order to ensure that the particular interval of temperature to be measured shall fall within the limits of the scale. In order to set the thermometer it is inverted and then gently tapped until the reserve mercury passes to what, in the upright position, is the

uppermost end of its reservoir. The thermometer is now returned to its normal position and warmed in a bath of water until the mercury thread has risen to unite with the reserve mercury. The bulb is next cooled in a bath whose temperature is about 2—3°C. higher than that which is to be observed. This means a temperature of about +2°C. if the freezing point of an aqueous solution is to be measured. Finally the excess of mercury is detached from the thread by a suitable jerk.

The large outer vessel is to be filled with a mixture of ice, water and an amount of salt sufficient to maintain a temperature about 5°C. less than that to be observed. Pure water is now run into the innermost tube until the bulb of the thermometer is well covered. The whole is then placed directly in the freezing mixture, either by removing the cover of the outer jar or by taking advantage of an aperture specially provided for the purpose. The water is continuously agitated by means of the ring-shaped stirrer until it has cooled almost to its expected freezing point. The cylinder is then removed from the freezing mixture and placed in the middle tube as shewn in the figure. The stirrer is now worked slowly and regularly up and down without rubbing the thermometer until the temperature has sunk ·5°—2°C. below the expected freezing point. The water is now made to freeze either by more vigorous stirring or, if this fails to produce the desired result, by the introduction of a small crystal of ice on the end of a fine glass rod inserted through the side tube. The crystal is taken off on to the stirrer and quickly immersed in the water, when freezing at once sets in. The stirring is maintained vigorously while the mercury thread of the thermometer is rising and is then slowed down to a regular rate of about one stroke per second until the reading of the thermometer has become absolutely steady. This reading is then noted, thousandths of a degree being estimated by the help of a hand lens. The value so obtained should be confirmed several times in order to arrive at the exact reading corresponding to the freezing point of pure water.

For the sake of practice in dealing with a solution 6·84 gm. of cane sugar are dissolved in 100 c.c. of water and sufficient of this liquid is placed in the freezing vessel to cover the thermometer to the same extent as did the water previously. The depression of the freezing point of this solution is ·372°C. and this value should be found consistently within one or two thousandths of a

degree in successive determinations. During this experiment it will be noticed that a solution, unlike pure water, has no constant freezing point. The sugar does not separate out during the freezing so that the remaining solution becomes gradually more and more concentrated, and as a result the temperature of the freezing mixture becomes more and more depressed. The value given above for the freezing point of the solution applies only if a small but distinct quantity of ice is allowed to separate out during the determination.

The molecular weight M of the cane sugar can be calculated from the equation:

$$M = E \cdot \frac{s}{\Delta L}$$

where E is a constant depending on the nature of the solvent and having for water a value of 1·86, s is the weight of the dissolved substance in grams, and L is the weight of the solvent in kilograms (if too much ice is allowed to separate out its weight should be substracted from the value of L.) Δ is the observed depression of the freezing point.

The solution we have used contains $^1/_5$ of a gram molecule of sugar in 1 liter of solvent. Urea in the same molecular concentration (1·20 gm. + 100 c.c. water) would give the same value for Δ. On the contrary, NaCl in the same molecular concentration (1·168 gm. + 100 c.c. water) would give a value almost twice (as a matter of fact about 1·8 times) as great, in consequence of its electrolytic dissociation to the extent of about 90 per cent.

The depression of the freezing point of blood amounts to ·58°C.[1] The osmotic pressure is proportional to the depression of the freezing point and at 18°C. amounts to almost 12 atmospheres in the case of a solution which has a value of Δ of 1°C. Such a high osmotic pressure cannot be measured directly as there are no semi-permeable membranes that could stand it without rupture. A value of Δ of as little as ·001°C. corresponds, therefore, to an osmotic pressure of 120 cm. of water. As we shall see immediately the osmotic

[1] An interesting exercise—and one that gives the student practice in dealing with body fluids—is to compare the depression of the freezing point of defibrinated blood that has been collected and exposed to air in the ordinary way with that of blood that has been shaken with CO_2. The increase in concentration of ionised molecules ($NaHCO_3$) that takes place when the blood takes up this gas is thus easily demonstrated.—*Translator.*

pressure of colloids amounts to only a small fraction of this value so that it follows that the depression of the freezing point of colloidal solutions must be immeasurably small.

EXERCISE 46.

Measurement of the Osmotic Pressure of Colloidal Solutions.[1]

The osmotic pressure of a colloidal solution is much easier to measure directly than that of a true solution because it is so much easier to prepare membranes which are semi-permeable to colloids. Collodion membranes are suitable for the purpose and they are used in exactly the same form as is described on pp. 97-98. In the case of the measurement of osmotic pressures it is not a question of observing the rate of passage of water through the membrane, as it was in the case of the experiments on the osmosis of water, but of determining the final definitive height to which the liquid ascends the glass tube above the level of the outside fluid. We will measure the osmotic pressure of a 1 per cent. solution of gelatin in pure water. The solution is placed in the collodion sac, which need not necessarily be completely filled, and pure water is placed outside. The glass tube is so supported in the stand that the outside water does not quite reach the level of the rubber cork. In winter it is necessary to set up the apparatus in a warm place as the solution becomes viscous when cooled. At the beginning of the experiment the tube should be blown into in order to cause the liquid to ascend and so to make it possible to test the tightness of the apparatus. It is further necessary to make certain, by raising and lowering the sac, that the liquid will move freely in the glass tube. The glass tube must be free from air bubbles. The steady state will have been reached after 24 hours, when it will be found that the pressure indicated is between 20 and 50 mm. of the solution above the level of the water outside. A second experiment should be carried out with an acidified solution of gelatin (50 c.c. of 2 per cent. gelatin solution + 2 c.c. of N. HCl + 48 c.c. of water). In this case N/50 HCl is used as the outer fluid. The osmotic pressure is now found to be greater, its value amounting to about 70 mm., shewing that the osmotic of a colloid

[1] Taken from the works of Jacques Loeb and S. P. L. Sörensen quoted in the footnote to the section entitled "Osmosis."

depends not only on its concentration but also on the nature of the medium in which it is dissolved. In the pure gelatin solution the reaction is usually in the neighbourhood of pH 6 and so is not far removed from the iso-electric point of gelatin (pH 4·7). At the iso-electric point the osmotic pressure of gelatin is a minimum. In strongly acid or alkaline solution the gelatin forms positively or negatively charged ions, respectively, and these approach, or even attain, a molecular degree of dispersion and so exert a greater osmotic pressure. Another factor which enters in to the phenomenon is the electro-static attraction between the positively charged gelatin ions and the negatively charged chlorine ions whereby the latter are prevented from diffusing through the membrane and so also help to increase the total osmotic effect. (See the later section on Donnan Equilibrium.)

Instead of gelatin solution a three-fold dilution of blood serum may be used. The diluting and outer fluid may be ·85 per cent. NaCl solution in one experiment and distilled water or N/50 HCl in a second. Again, the osmotic pressure will be found to be influenced by the nature of the medium, and the influence will be found in many particulars to run parallel to that of the various salts on the rate of osmosis of water through a gelatinised collodion membrane (see p. 99).

Blood serum diluted three times with ·85 per cent. NaCl solution gives an osmotic pressure against the same salt solution of about 143 mm. diluted five times it shews a pressure of about 75 mm. These values must be taken merely as fixing the order of magnitude of the pressures, as the osmotic pressure of colloids is very susceptible to changes in the salt concentration and the reaction of the solution.

The above are simply demonstration experiments. For accurate quantitative work more complicated arrangements for the maintenance of a constant temperature, etc., are necessary.

VIII.

Swelling, Viscosity and Gel Formation.

The power of combining with water expresses itself in the case of a substance that is molecularly dispersed in solution by its power of passing spontaneously into solution when brought into contact with water and by its property, which is assisted by shaking, of distributing itself uniformly over the whole bulk of the solvent. In the case of a spontaneous colloid similar relationships obtain, but in this case the effect of shaking is much more marked as the spontaneous diffusion is very sluggish; and further, the process of subdivision often stops short of the condition of molecular dispersion and leads to the formation of aggregates of molecules—micellae—which are visible under the ordinary or at all events under the ultra-microscope. An intermediate type of behaviour is shewn by those substances which are associated in solution to form polymers composed of two or more molecules. If we have to account for the incompleteness of the dispersion, in the present condition of our knowledge only one assumption appears possible. Solubility is the expression of an affinity between the dissolving substance and the molecules of the solvent water; but at the same time there exists the possibility of an affinity between the solute molecules themselves. Now in the case of a molecule with a complicated structure it might happen that one part of the molecule—that containing a –COOH group, for example—has a great affinity for water while another part of the same molecule might have more affinity for molecules similar to itself than for water molecules. Such a molecule might be said to be to a certain extent soluble in water at one end and to be insoluble at the other. In this way there will arise complexes of molecules with which a certain quantity of water will be associated, i.e. which will be to a greater or less extent hydrated; and which, even in the case of complexes composed of the same numbers of solute molecules, will occupy different volumes according to the degree of hydration.

One can distinguish two types of such systems. The micellae may remain as separate particles floating in the solvent, or they

may, if they are sufficiently numerous, adhere under certain circumstances, to form filaments, or a network, or even a closed honeycomb structure. In this latter case the micellae have formed a continuous mass and it is the solvent that is now subdivided into discrete droplets, whereas it is usually the colloidal particles that are so separated. Such a system will no longer run in drops; it forms a jelly. The solidity of such a jelly will depend on the consistency of its network. In general the jelly will be more solid, other things being equal, the less the degree of hydration of the substance of its framework. Furthermore each cell of the honeycomb is analogous to a completely closed collodion sac, inasmuch as its walls are permeable to simple molecules and ions but impermeable to the more complex structures. If therefore, the liquid inside the cell contains a colloid in a condition of fine or of molecular dispersion the osmotic pressure of these non-permeating molecules, and of any ions that may be retained by them by electro-static attraction, must shew itself in the same way as in the case of the collodion sac. Water will be drawn into the cell until the difference of osmotic pressure inside and out is just balanced by the elastic tension of the walls. These concepts enable us to account for the setting of a colloidal solution to form a gel, for the power of swelling possessed by such a gel and for the degree of viscosity exhibited by colloidal solutions when still in the fluid condition. All ions have a high capacity for combining with water so that all factors that increase the electric charge on the colloid former, that is its degree of ionisation, will increase its degree of hydration, and the colloid particles will swell at the expense of the surrounding fluid. If the colloid is in the form of discrete particles this restriction of the relative volume occupied by free water molecules will, according to a law formulated by Einstein, increase the viscosity: if the colloid is already in the form of a gel the same factors will increase the degree of swelling, and if the colloid has a consistency intermediate between that of a freely flowing liquid and that of a jelly an increase of ionisation will favour the retention of the fluid condition, while a decrease of ionisation will produce a tendency towards gel formation.

The most potent factor that determines the degree of ionisation of the colloid-former is the simultaneous presence of other ions in the solution, and among the ions that occur naturally in the living organism those of $H^{\cdot}$ and OH' are again distinguished by their

special activity. The iso-electric point of the colloid always forms a characteristic point, marked, in the case of a liquid, by a minimum of viscosity, and in the case of a gel by a maximum tendency to solidification and by a minimum of swelling.

The activity in this respect of any particular ion that may be in the solution will vary considerably according to a number of its properties: on the sign of its charge as compared with that on the colloid, on its valency, its position in the Hofmeister series and on its "adsorbability." Most of the ions that possess a well-marked pharmacological action are strongly adsorbed and enter into serious competition with the $H^{\cdot}$ and OH' ions. In addition to ions, surface-active non-electrolytes shew a quite appreciable degree of activity, but their mode of action is not yet fully explained. The following experiments are concerned chiefly with the effects produced by $H^{\cdot}$ and OH' ions.

If the acidity of a gelatin solution is gradually increased by the regulated addition of HCl a point of minimum swelling is found at a very low concentration of HCl corresponding to the iso-electric point. As the requisite quantity of HCl is very small it is more convenient to increase the acidity by means of buffer solutions than by the addition of the free acid. As the concentration of HCl is increased the gelatin becomes more and more ionised and so swells to a larger and larger volume, until finally the antagonistic effect of the Cl ions becomes sufficiently great to cause a diminution of the swelling. Gelatin therefore shows also a point of maximum swelling at a particular concentration of HCl.

EXERCISE 47.

The Points of Maximum and Minimum Swelling for Gelatin.

The optimum concentration of HCl for swelling.[1]

A series of solutions is made up as follows:

No.	1	2	3	4	5	6	7	8	9	10
1 N.HCl c.c..	16	8	4	—	—	—	—	—	—	—
·1 N.HCl c.c.	—	—	—	20	10	5	2·5	1·25	·62	·31
Distilled water c.c. .	4	12	16	0	10	15	17·5	18·75	19·38	19·69

[1] Quoted from *Chiari, Biochem.* Zeitschr. **33**, 167. 1911.

Strips of as nearly as possible equal size, 5 cms. × $^1/_2$ cm., are now cut from a sheet of gelatin at least 1 mm. thick, and one such strip is placed in each tube. After 24 hours the lengths of the strips will be found to be as follows (provided that the original gelatin sheet was not too thin and they have not fallen to pieces):

No.	1	2	3	4	5	6	7	8	9	10
	6·4	6·6	6·7	7·4	8·3	8·3	7·6	7·0	6·1	5·3 cms.

The optimum swelling occurs therefore between ·05 and ·025 N.HCl, which means a pH of 1·3 to 1·6 in the outer fluid.

But at the point of equilibrium the pH of the outer fluid is not the same as that of the internal liquid of the gel (see the later section on Donnan equilibrium) which, when pure gelatin is used, has been shown to have an accurately reproducible value of 3·2.[1]

In contrast with this point of maximum swelling there exists a point of minimum swelling which occurs at the iso-electric point of gelatin (pH = 4·7). This is best demonstrated by weighing the gelatin. Pieces of gelatin of exactly equal weights (in our case 1·529 gms.) are placed in five suitable vessels of about 100 c.c. capacity, which are then filled respectively with the following five solutions:

	1	2	3	4	5
N/10 sodium acetate	5	5	5	5	5
N/10 acetic acid .	·31	1·25	5	20	80
Water . . .	94·69	93·75	90	75	15

After 24 hours' soaking, the pieces of gelatin are taken out, dried on filter paper and again weighed. In a particular experiment the result was:

	1	2	3	4	5
Weight of gelatin .	11·0	8·9	7·9	8·3	15·7
pH . . .	5·8	5·2	4·6	4·0	3·4

The lower row of figures gives the pH value as calculated from the compositions of the solutions. The minimum swelling occurs at the iso-electric point (pH = 4·7).

It should be emphasised that the point of maximum swelling occurs at pH = 3·2, while the minimum occurs at pH = 4·7, so that

[1] Jacques Loeb, *Proteins and the Theory of Colloidal Behavior*. New York, 1922.

quite a small change of reaction suffices to change the gelatin from one extreme condition to the other. This is yet another example of the fact that it is impossible to make any statement regarding the relation between the condition of a colloid and its chemical environment without taking into account the exact pH value.

EXERCISE 48.

Estimation of the Internal Friction (Viscosity) of a Solution.

The internal friction of a fluid is best determined by means of the viscosimeter of Wilhelm Ostwald. The values obtained are relative to that for pure water which is taken as unity. Since the viscosity is influenced very considerably by changes of temperature it is necessary to carry out the measurement in a water bath. The same quantity of liquid must always be used in the viscosimeter and the suitable volume must be determined for each instrument. Water is run from a pipette into the wider tube *d* (see fig. 14) until the bulb *c* is just full. The water is then blown from tube *d* into the other limb until it stands at the mark 1; it should then still reach round to the bulb *c*. Once the volume of liquid which fulfils this condition has been determined it should be exactly adhered to in all measurements made with the particular instrument. Usually 5 c.c. are required. Time is now measured with a stopwatch reading to $^1/_5$ of a second, and when the meniscus passes the upper mark 1 the watch is released; it is stopped when the fluid just passes the lower mark No. 2. The liquid is again forced up into the limb *a*, and the observation is repeated until constant values are obtained. This is particularly necessary in dealing with gelatin solutions, as these only slowly acquire their definitive viscosity after a change of temperature.

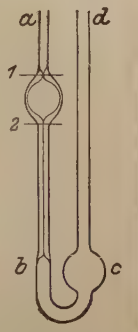

FIG. 14. Viscosimeter $^1/_6$ natural size.

At first a series of experiments should be carried out in order to establish the value for pure water at the temperature at which the main observations are to be made (35° C.).

The following will serve as exercises with the method:

1. The influence of "h" on the viscosity of gelatin.

A viscosimeter is chosen in which the time of flow for pure water is $^3/_4$—1 minute measurable to $^1/_5$ of a second.

3 gm. of pure gelatin are dissolved in 100 c.c. of distilled water with warming and vigorous shaking. Separate 10 c.c. portions of the solution are then diluted while still warm with 5 c.c. of the following solutions respectively:

1. ·33 N. HCl.
2. ·1 N. HCl.
3. ·02 N. HCl.
4. ·01 N. HCl.
5. ·001 N. HCl.
6. Distilled water.
7. ·005 N. NaOH.
8. ·1 N. NaOH.

The viscosity of each of these mixtures is then determined at 35° C., each observation being repeated until values agreeing to within $^2/_5$–$^4/_5$ of a second are obtained. The exact result of the experiment depends on the degree of purity of the gelatin employed, but the following will serve as an example:

Calibration with distilled water 57 seconds.

Solution	1.	120 seconds.
,,	2.	144 ,,
,,	3.	106 ,,
,,	4.	103 ,,
,,	5.	108 ,,
,,	6.	108 ,,
,,	7.	109 ,,
,,	8.	139·8 ,,

The maximum viscosity therefore occurs in solution 2, and the minimum in solution 4.

If the pH of each of the mixtures is determined by the method given on page 52 it is found that it is this solution No. 4 whose reaction is nearest to that of the iso-electric point of gelatin (pH = 4·7). It is also found that between tubes 3 and 6 the pH value changes only slightly—from about pH 5 to pH 4. This is due to the circumstance that free HCl, and not a buffer solution, has been used to change the reaction, and that at these low concentrations the acid is very largely combined with the gelatin and with any small quantities of mineral salts that it may contain. This accounts for the small differences of viscosity between tubes 4, 5 and 6. The contents of tube 2 are much more acid, pH about 2·5, and of tube 1 still more acid, the pH being about 1·5.

The alkali also increases the viscosity, but not to the same extent as does the acid.

2. Demonstration of the effects of salts on the viscosity of gelatin.

The effect of salts on the viscosity of iso-electric gelatin is very slight; it is better, therefore, to choose a strongly acid or strongly alkaline solution as a starting point.

In a first experiment to a mixture of 90 c.c. of 3 per cent. gelatin + 1 c.c. of 1 N. HCl, 9 c.c. of distilled water are added; this gives a solution of the same composition as No. 2 of the previous series, which gives a time of outflow in the viscosimeter of about 140 seconds. A second mixture is now made containing the same quantities of gelatin and acid, but 9 c.c. of ·5 M. NaCl solution in place of the distilled water. The time of outflow of this solution will be found to be about 105 seconds—almost the same value as was given by iso-electric gelatin.

In the presence of large quantities of neutral salts the viscosity of gelatin is therefore but little influenced by the hydrogen-ion concentration.

By using smaller concentrations of salt it is possible to show that the various neutral salts differ in the intensity of their actions.

The following experiment is carried out:

To successive separate mixtures of 90 c.c. of 3 per cent. gelatin + 1·0 c.c. of 1 N. HCl the following solutions are respectively added:

1.	9 c.c. M/20 NaCl	time of outflow	125 seconds.
2.	9 c.c. M/40 $CaCl_2$	,,	125 ,,
3.	9 c.c. M/40 Na_2SO_4	,,	118 ,,
4.	9 c.c. M/60 $AlCl_3$	,,	125 ,,
5.	9 c.c. distilled water	,,	139 ,,

From this experiment it is seen that all the salts diminish the viscosity of the acid gelatin—that is, bring its value nearer to that of the iso-electric substance. But in equivalent concentration the effects of the various salts are different in degree. In the acid solution the gelatin has a positive charge, so that it is only the negative ions that influence it. It is seen that the divalent sulphate ion of Na_2SO_4 has a more powerful influence than the univalent chlorine ion of NaCl. But the nature of the cation is a matter of indifference. The three chlorides NaCl, $CaCl_2$, $AlCl_3$ in equivalent

concentrations all produce the same effect, since then the concentration of chlorine ions is the same in each case.

On the other hand NaCl and Na_2SO_4 would have equal effects on alkaline gelatin, while that of $CaCl_2$ would be more powerful.

EXERCISE 49.

The Optimum Reaction for the Solidification and for the Development of the Opacity of Gelatin.

As already mentioned in the chapter on swelling (p. 107), we regard a jelly as a two-phase system made up of a honeycomb-like network composed of a relatively high concentration of the colloid with but little water enclosing in its pores a more dilute solution in which the colloid has a finer or even molecular degree of dispersion. A jelly differs therefore from a fluid solution of a hydrophil colloid merely in the circumstance that the more solid phase of the system, the phase richer in the colloidal material, is not in the form of discrete particles, but consists of this connected network. The solidification of a fluid to form a jelly consists in the union of such particles to form intercommunicating strands. All the factors which lead in the case of a simple solution of a hydrophil colloid to an increase of the disperse phase at the expense of the liquid phase must therefore necessarily increase the tendency to gel-formation. Among such factors we again include the presence of ions—particularly those of hydrogen. We shall restrict our investigations to a study of the influence of hydrogen ions on the process of gel-formation.

From the outset it is necessary to realise the apparently paradoxical fact that in gelatin solutions of equal concentrations the point of minimum viscosity, which is determined by the concentrations of such substances as influence the degree of dispersion of the colloid, coincides with that at which the solidification most readily takes place. That this must be so will be clear when it is remembered that the point of minimum viscosity, when it is determined at such a temperature that no trace of the network is present, indicates that the concentration of colloid in the fluid phase is a minimum; while the point at which gelation most readily occurs marks the condition of greatest concentration of the colloid in the solid phase. These two points must of necessity coincide.

It follows that a solution containing a given concentration of gelatin will solidify the more readily on cooling the less its viscosity at a temperature at which it is still permanently fluid.

The following mixtures are made up in test-tubes:

No.	1	2	3	4	5	6	7
N/10 sodium acetate c.c. . .	1	1	1	1	1	1	1
N/10 acetic acid c.c.	0	·06	·25	1	4	—	—
N. acetic acid c.c.	—	—	—	—	—	1·6	6·4
N. caustic soda c.c.	·05	—	—	—	—	—	—
Water c.c. . .	6·95	6·94	6·75	6	3	5·4	·6
pH (approx.) .	8	5·6	5·0	4.6	4·0	3·4	2·8

To each tube is added 3 c.c. of a warm fluid solution of 10 gms. of gelatin in 100 c.c. of distilled water. The tubes are then all warmed for a few minutes in a water bath at 50° C., then cooled in a bath at room temperature, and finally set in a rack either in the room or in the ice-chest. From time to time the tubes are tilted in order to detect the onset of solidification. If the solidifying point is taken to be that at which the solution will no longer flow when the tube is tilted, the time taken to arrive at this condition will vary according to the temperature, but for the several tubes will be somewhat as follows:

Tube No.	1	2	3	4	5	6	7
Time required for solidification (mins.) . .	19	17	16	14	19	25	very long

The observation may be repeated as often as may be desired with the same solutions by remelting the jellies. The most rapid gelation occurs in tube 4 (pH=4·6), which is nearest to the iso-electric point of gelatin (pH=4·7). When the solidification is complete it will be noticed that in this tube containing the iso-electric mixture the gelatin shows a distinct opalescence resembling that of a dilute mastic sol. At least traces of this opalescence will also be observed in the neighbouring tube on the right and in one or even two tubes to the left. The appearance is due to the great difference in the refractive indices of the disperse phase and the dispersion medium—a difference which in turn is due to their respectively great and small concentrations of gelatin, at the iso-electric point. This important phenomenon of the maximum opacity of gelatin is quite unintelligible without a knowledge of the pH of the solutions, and indeed was formerly completely overlooked.

IX.

Electro-phoresis and Electro-endosmose.

A particle suspended in water will in general possess an electric charge with respect to the fluid, and if it is free to move it will travel to the pole of opposite sign when a potential difference is applied.[1] If, however, the particles are held still, say by causing them to adhere in large numbers to the pores of a diaphragm arranged as a barrier across the liquid, the water will pass through this diaphragm in the opposite direction.

The presence of the charge on the particles is explained by the adsorption of ions; the layer of adsorbed ions constituting the inner layer of an electric double layer, the outer layer of which is formed by ions of opposite charge which are held by electrostatic attraction and not by adsorption.

With regard to the sign of the charge on the particles we can at present make the following statements: If the particles possess to a marked extent the chemical properties of an acid (silica, resin acids), of a base (clay), or of an ampholyte (proteins), the sign of their charge will be such as would be expected from the mode of ionisation; negative in the case of an acid, positive in the case of a base, and either positive or negative according to the value of h in the case of an ampholyte. Particles which do not show these marked chemical characteristics (colloidal metals, cellulose, collodion) are usually found to possess a negative charge; blood charcoal is found to behave as an ampholyte, while sugar charcoal and gas carbon behave as acids.[2]

All negative charges are diminished and sometimes even reversed by increase of hydrogen-ion concentration and by trivalent cations. All positive charges are diminished, and under certain circumstances reversed by increase of the OH′ ion concentration and by strongly active anions such as thiocyanate, etc. Negative charges occur most commonly.

[1] One ordinarily speaks of sending a current through the liquid, but it is really the fall of potential per cm. that is the determining factor—the strength of the field and not the strength of the current.

[2] Bethe and Toropoff, *Zeitschr. f. physik. Chem.* **88**, 686, 1914 and **89**, 597, 1915; Gyemant, *Kolloid Zeitschr.* **28**, 103, 1921; Umetsu, *Biochem. Zeitschr.* **135, 442, 1923.**

The negative charge of cellulose and of agar is only diminished and never reversed by $H^{\cdot}$ and $Al^{\cdots}$ ions. In the case of kaolin and gold sol the sign of the charge can be reversed by $Al^{\cdots}$ ions, but not by $H^{\cdot}$; but both of these ions will reverse the negative charge in the case of amphoteric substances such as proteins and in the case of blood charcoal.

All proteins belong to the class of reversible ampholytes.

EXERCISE 50.

The Electric Cataphoresis of Hæmoglobin.[1]

The apparatus used is that shown in fig. 15. It is on the principle devised by Landsteiner and Pauli, but is considerably modified, particularly with regard to the arrangement of the electrodes. Spaces 2 and 4 are to be filled with the "side fluid," and space 3 with the "middle fluid," whose preparation will now be described.

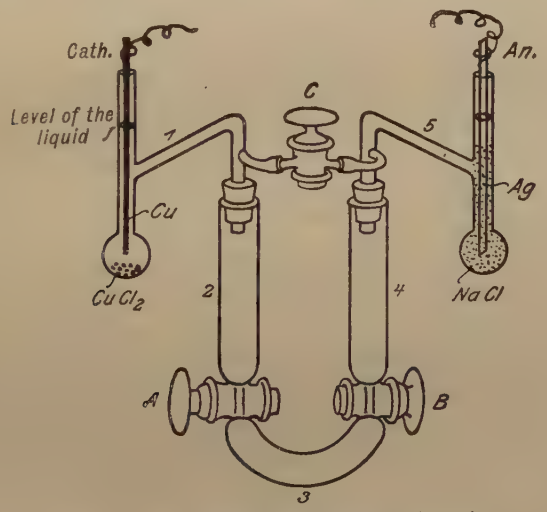

FIG. 15.—Apparatus for Electrophoresis.
3/10 natural size.

1. Preparation of the middle fluid.

Five c.c. of defibrinated blood (of sheep, horse, bullock, rabbit or man) are diluted with 100 c.c. of ·85 per cent. NaCl solution and then rapidly centrifuged. The NaCl solution is now sucked off as

[1] L. Michaelis and H. Davidson, *Biochem. Zeitschr.* **41**, 102, 1912.

completely as possible and the corpuscles are dissolved in 100 c.c. of distilled water. The object of this washing and diluting is to reduce the buffering power of the blood itself so that it shall affect as little as possible the reaction of the buffer solutions that must subsequently be added to it. To 30 c.c. of this solution are now added 3 c.c. of M/3 primary sodium phosphate and ·2 c.c. of M/3 secondary sodium phosphate (for the mode of preparing these solutions see p. 85). Finally, ·5 gm. of sugar is dissolved in the liquid in order to increase its specific gravity to a certain extent.

2. Preparation of the side fluid.

This is the same solution as the middle fluid with the blood and sugar omitted, and is prepared by adding 9 c.c. of M/3 primary sodium phosphate and ·6 c.c. of M/3 secondary sodium phosphate to 90 c.c. of distilled water.

The pH value of this solution is determined on a 10 c.c. sample by the method described on page 52, using p-nitrophenol as indicator. The result will be found to be approximately pH = 5·5.

The side tubes with their rubber stoppers are now removed from the apparatus, the taps A and B are carefully dried and greased, and then with these taps open the middle fluid is run in until space 3 is completely filled and the fluid extends through the bores of the taps a little way into chambers 2 and 4. When all air bubbles have been removed the taps are closed, and chambers 2 and 4 are washed out first several times with water and then with some of the side fluid, which is finally used to fill both chambers completely. At this stage it is convenient to clamp the apparatus temporarily in a stand in the position shown in the figure in order to facilitate the replacement of the side tubes and their stoppers without permitting air-bubbles to remain in any part of the tube. The side tubes themselves are next completely filled with the side fluid from a 5 c.c. or a 10 c.c. pipette, the same care being taken to remove air-bubbles by suitably tilting the apparatus. The fluid should stand at the level shown in the figure. The tube can now be finally clamped in position, with the tap C closed for the time being.

The depolarising materials must now be supplied. Into the limb that is to serve as anode about 1 gm. of solid NaCl or KCl is shaken, care being taken to ensure by moving a wire up and down in the solution that the whole of the salt does not sink to the bottom,

but that some of it is dissolved in the upper liquid. Into the other limb about ·2 gm. of $CuCl_2$ is dropped, and this is allowed to sink right to the bottom of the tube.

The anode itself consists of a long strip of plain silver foil inserted into the side tube that has received the KCl; the cathode consists of a copper wire which has been coated with paraffin wax so that only the extreme end that comes in contact with the copper chloride solution remains bare. (The covering on ordinary insulated wire is not sufficient for this purpose.) The apparatus is now connected through a lamp for safety to a 110 or 220 volt. direct current lighting circuit in such a way that the silver is connected to the positive pole (the one that reddens litmus), and the copper is connected to the negative pole (the one that turns litmus blue).

Before the current is switched on tap C is opened temporarily in order to bring the fluid to the same level in each tube. This tap is then closed and A and B are opened and the apparatus is allowed to stand for 5 minutes in order to enable it to be ascertained that the surface of separation between the hæmoglobin solution and the colourless fluid on both sides remains sharp and does not become displaced, and that the taps A and B are quite tight and do not allow fluid to leak to the outside. If these conditions are fulfilled the current is turned on. After half-an-hour it will be found that the whole column of hæmoglobin has shifted several millimeters towards the cathode; the sharp surface of separation between the red and the colourless solutions has risen on the side carrying the copper electrode and fallen on that of the silver one.

If the hæmoglobin solution shows layers and striations these are not the effect of the electric current, but are due to disturbances caused by heating or by vibration. The addition of sugar to the middle fluid greatly reduces its sensitiveness to such extraneous influences.

When the observation has been carried on for a sufficient length of time the current is switched off, taps A and B and then C are closed, and the electrodes are withdrawn. The side tubes are then removed—that which contained the copper wire first—by closing their open apertures by means of a finger and then rapidly lifting their stoppers in such a way as to prevent their contents from mixing with the fluid in chambers 2 and 4. These fluids are now removed separately by means of a pipette, care being taken not to disturb any hæmoglobin that may be at the bottom, and a

redetermination of the pH is made in order to make sure that it has not changed during the experiment. This should be the case within a margin of ±·1.

If the electrometric method is used the pH of the coloured middle solution can also be determined at the beginning and the end of the experiment.

The result of the observation is to show that at pH 6 hæmoglobin wanders to the cathode; that is, it is positively charged.

If the hæmoglobin is dissolved in a solution of pH about 8 it will be found to migrate to the anode. The following solutions are recommended:

As middle fluid: 30 c.c. of diluted blood, prepared as before, +·1 c.c. of M/3 primary phosphate + 3·0 c.c. of M/3 secondary phosphate + ·5 gm. sugar.

As side fluid: 90 c.c. of distilled water + ·3 c.c. of M/3 primary phosphate + 9 c.c. of M/3 secondary phosphate. pH = a little greater than 8.

The iso-electric point at which absolutely no movement would be observed is pH = 6·8.

If there happens to be any of the denaturated albumin left over from exercise 25 (p. 66) this may also be used for a similar experiment. The movement of the cloudy fluid can be followed in the same way as that of hæmoglobin solution. Suitable liquids for use in this case are:

For movement towards the anode:	Middle.	Side.
1 N. sodium acetate solution, c.c.	·5	1·5
·1 N. acetic acid	·5	1·5
Diluted albumin solution	30·0	water 90·0

For movement towards the cathode:	Middle.	Side.
·1 N. sodium acetate solution c.c.	·2	·6
1 N. acetic acid	1·0	3·0
Diluted albumin solution	30·0	90·0

EXERCISE 51.

The Quantitative Estimation of the Rate of Cataphoresis.

The apparatus described in the previous exercise can be used only as a null instrument; that is, for the determination of the pH at which the movement becomes reversed in direction. It is not adapted for the measurement of the absolute rate of the movement

under the influence of a given strength of electric field, because in consequence of the varying cross sectional area of the path of the current (side tubes, bores of the taps) the field is not uniform, so that one cannot calculate the strength of the field in terms of the potential gradient per cm. In fig. 16, however, is shown a form of apparatus that will serve for such absolute measurements, and that is also suitable for use as a null instrument. The tubes and taps are of uniform diameter throughout. The current is not brought direct to the tube by metallic electrodes, but by U-tubes of glass filled with KCl agar jelly made by boiling 3 gms. of agar and 40 gms. of KCl in 100 c.c. of water. One limb of each agar tube is inserted into one of the open ends of the cataphoresis apparatus, while the other limbs are made to dip into flasks of 10 per cent. $CuSO_4$ solution in which are also immersed spirals of bare copper wire to serve as electrodes. The agar tubes should be supported in a stand in such a position that their lower ends reach right down to the sharp bends in the limbs of the cataphoresis tube itself, and they must be made of the appropriate width. On the one hand they should be of such diameter that there is not merely a capillary space between themselves and the limbs of the cataphoresis tube, but room for the formation of a definite water meniscus; on the other hand they must not be excessively narrowed, as this would unnecessarily increase their resistance, and the calculation of the strength of the electric field assumes that the resistance of the agar leading-in tubes is negligible compared with that of the cataphoresis tube itself. Each agar tube may be slightly narrowed only at its extreme tip where it reaches the angle of the side limb of the main apparatus.

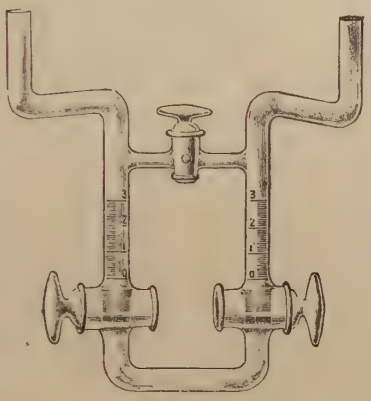

FIG 16.

Both limbs of the cataphoresis tube are provided with a scale of millimeters, the graduations of which must be carried sufficiently far round the circumference of the tube to enable readings to be made without errors due to parallax. A suitable material for the experiment is the sol of gum mastic prepared as directed in

Exercise 4; for this purpose a very turbid, almost milky solution should be used. In order to make it somewhat denser about 1 per cent. of sugar is dissolved in it, and an addition of, say, $^1/_{20}$ of its volume of phosphate mixture " 8 " is also made to it. As side fluid a 20-fold dilution of the same phosphate mixture " 8 " is used without the addition of either sugar or mastic. The object of using the phosphate is to maintain a definite value of pH, for the rate of

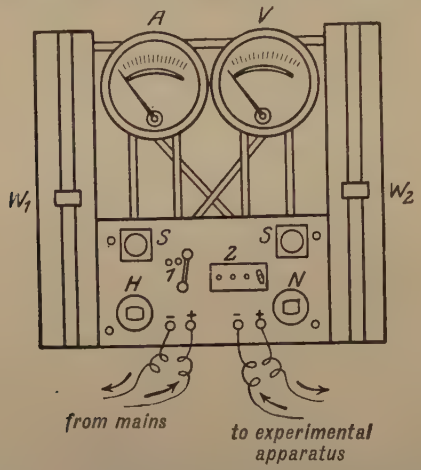

FIG. 17.—Schematic Diagram of a simple Switchboard for Electrophoresis Experiments.

The current from the mains (110 or 220 volts, direct) is brought to the two terminals indicated; that to the electrophoresis tube is led off from the remaining terminals. W_1 and W_2 adjustable resistances of the sliding type: one gives a coarse and the other a fine adjustment. A is the ampèremeter and V the voltmeter, both of which have three ranges of reading, so that the ampèremeter can be read accurately between 3 and ·001 amps. and the voltmeter between 220 and ·001 volt. 1 is the range setting for the ampèremeter and 2 that for the voltmeter. S S are fuses. H is the main switch for the supply current, N controls the current through the side circuit to the electrophoresis tube.

the cataphoresis depends on the hydrogen-ion concentration, and also to impart the same conductivity to both middle and side fluids. In the absence of the salt the conductivities of these fluids would be determined solely by the traces of salts present as impurities, and so might easily be unequal. In other respects the procedure is the same as that adopted in the preceding exercise. The time at which the level of the mastic solution reaches the zero point of the graduations is noted in order to obtain a starting point for the measurement of the rate of movement. The strength of the electric

field is obtained by dividing the value of the externally applied electromotive force by the total distance in cms. traversed by the current in passing from the end of one agar leading-in tube, round the U-tube of the apparatus, to the end of the other agar tube.

The voltage applied to the apparatus is best regulated by means of a switchboard from which any desired fraction of the total voltage of the lighting mains may be obtained. The most convenient potential differences to employ are those of 50, 100 or 200 volts. A rocking commutator should be included in the circuit.

This form of migration apparatus has the advantage that its depolarising system will work in either direction so that it is possible to reverse the current during an experiment in order to obtain movement in the opposite direction. With the arrangement previously described it was necessary to send the current through only in the prescribed direction.

The result of such an experiment as we have described was found to be as follows: Total distance traversed by the current between the agar tubes, 32 cm.; externally applied potential difference, 100 volts.; the field strength was therefore 3·2 volts./cm. The line of separation of the mastic solution arrived at the zero line of the graduation about 8 minutes after the closure of the current. After this the level of the liquid shifted at the rate of about 1 mm. in 2 minutes, which, calculated for a field strength of 1 volt./cm., comes to be ·157 cms. per minute, or $2{\cdot}62 \times 10^{-3}$ cm. per second. It was easy to show that on doubling the applied potential difference to 200 volts. the rate of migration is doubled, and that it is halved as a result of a halving of the potential. A reversal of the direction of the current leads to a change in the direction, but not in the rate of the movement. For all these manipulations it is essential that the surface of separation between the mastic solution and the clear side fluid should remain sharply defined for some considerable time.

EXERCISE 52.

Observation of the Cataphoresis of Red Blood Corpuscles under the Microscope.[1]

For the correct observation of electrocataphoresis in microscopical preparations a special suitably constructed chamber is essential. If the movement is observed in a thin film of suspension between

[1] Simplified and modified from R. Höber, Pflüger's Arch. **101**, 607, 1904.

an ordinary cover slip and slide very deceptive appearances present themselves, as will be readily understood from the theory of the process, which is as follows: When a difference of potential is established between the ends of a microscopic chamber the primary effect is a movement of the suspended particles with respect to the fluid; this is the true cataphoresis that we wish to observe. But the surface layers of water that are in contact with the upper and lower glass surfaces will also show a drift in the electric field, because water takes on a charge, usually of positive sign, when in contact with glass. If we consider the case of a closed chamber it is evident that, as a result of this drifting of the upper and lower layers of water in the same direction, there must be established a compensatory back flow in the middle layers. If, therefore, we regard the whole mass of water as made up of a large number of lamellæ, under the influence of the applied electromotive force the upper and lower of these lamellæ will be moving towards the cathode and the middle lamellæ towards the anode, and between these two positions all possible transitional forms of movement will be found. The rate of movement of the liquid towards the cathode will therefore decrease as we observe layers more and more deeply situated in the chamber, and at a certain depth will possess a value of zero; the movement will then be reversed in direction, and will gradually increase in rate until in the middle layers of the liquid a maximum velocity in the direction of the anode is observed. These conditions are repeated symmetrically in the lower half of the chamber. The movement of the water becomes added algebraically to the true cataphoretic migration of the particles, so that it is only in the layers where the water is stationary that the uncomplicated movement of the particles can be observed. According to v. Smoluchowski[1] the two layers that fulfil this condition are those whose distances from the upper glass surface are given by the formula:

$$d\left(\frac{1}{2} \pm \sqrt{\frac{1}{12}}\right)$$

Where d represents the total depth of the chamber. This means roughly the layers $^1/_5$ and $^4/_5$ d from the upper surface.

If the rate of cataphoresis is therefore to be observed accurately

[1] In Grätz' *Handbuch der Elektrizität*, Bd. 2, 1912; reprinted unchanged in the 2nd edn. 1920.

it is essential that the suspended particles should not settle appreciably and so pass into other layers of the fluid. This condition can hardly be attained in the case of blood corpuscles, which are consequently not so suitable for this experiment as are bacteria such as Staphylococcus; these show quite well the opposite directions of movement at the sides and in the middle of the chamber. The simplest form of chamber to use for the purpose is that shewn in figure 18. It is made by cementing to an ordinary slide by means

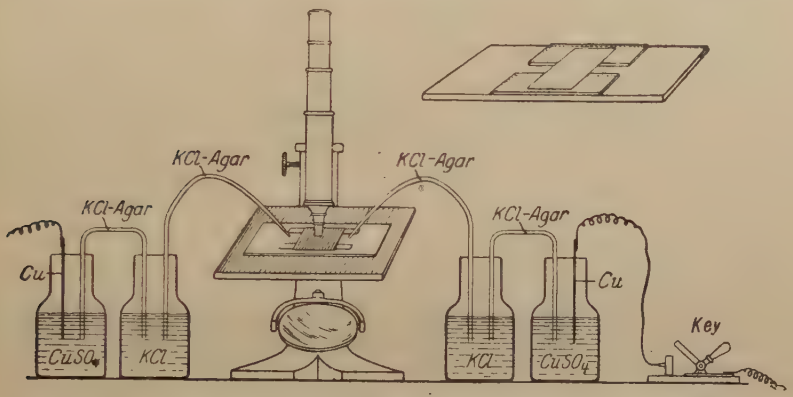

FIG. 18.—Electric Cataphoresis. The upper figure on the right represents the microscopic chamber on a larger scale.

of Canada balsam (or paraffin wax) two strips cut from not too thin a coverglass (·1–·2 mm. thick). After several days' drying excess of balsam is removed. The chamber can now be filled with the fluid under investigation, and covered by a slip beyond whose edges the liquid should extend for a certain distance on each side.

In these projecting drops of fluid are immersed electrodes consisting of suitably bent glass tubes drawn out to fine points and filled with a 10 per cent. solution of NaCl or KCl in 3 per cent. agar. The free ends of the agar tubes dip into flasks containing a salt solution of the same or of greater concentration. For experiments of short duration plain electrodes of copper wire leading through a key placed conveniently near the microscope to direct current mains at 110 volts may be inserted directly into these salt solutions, but it is preferable to connect the salt solutions through syphon tubes also filled with salt agar to flasks of 10 per cent. copper sulphate solution which themselves carry the copper electrodes.

As experimental material any defibrinated blood diluted 50-fold with ·85 per cent. NaCl solution, or a quite dilute suspension of staphylococcus may be used.

The fluid is watched, preferably with an eye-piece carrying a cross wire or micrometer scale, until the streaming of the corpuscles has ceased. If now the circuit is closed the corpuscles which are situated in the plane equidistant from the top and bottom of the chamber will be seen to move energetically towards the anode, and to seek the same pole when the current is reversed.

If a bacterial suspension is used it is particularly easy to see that the uppermost and lowermost layers are moving in a direction opposite to that shown by the middle layers. For the determination of the rate of the true cataphoresis of the particles the microscope must be focussed by means of a micrometer screw on the layers at depths of $\cdot 2d$ and $\cdot 8d$. In the case of diluted blood the corpuscles settle too rapidly for observations to be made on the uppermost layer, and they adhere too firmly to the slide to enable the movement of the lowermost layer to be observed. But the important layers can be investigated, and also what for purely qualitative comparisons is of almost equal value, namely, the rate of movement in the middle layer.

The addition of a trace of aluminium chloride to the corpuscle suspension will cause a reversal of the direction of the movement.

It is necessary to add only an ultracondenser to this equipment in order to make it suitable for the observation of colloidal solutions of mastic, proteins, etc.

EXERCISE 53.

Microscopic Observation of the Electric Cataphoresis in an Oil Suspension.

The arrangement used in the preceding exercise is not convenient for the simple calculation of the field strength, as the resistance presented by the agar tubes is not negligible compared with that of the chamber itself. Its great advantage lies in the efficiency of its depolarising system which is capable of carrying even heavy currents. For objects which do not require such a powerful field another arrangement may be used in which the field strength may be readily calculated. But the apparatus we are about to describe is not adapted for use at high voltages because the depolarisation is not sufficiently effective to prevent the formation of gas bubbles

on the electrodes in the chamber. The agar tubes are dispensed with and copper wires are used directly as electrodes. They are bent into a shape somewhat resembling that of the trolley-pole of a tramcar in such a way that the shorter straight arm will close more or less completely the side aperture of the chamber between slide and coverslip. These ends of the wires are heated in a flame before use in order to coat them with a layer of oxide; they are then clamped in position. In a feebly conducting fluid a potential difference of 10 volts may be applied to them without the formation of gas bubbles. A suitable experimental material is the emulsion obtained by shaking aniline with water. The necessary potential difference of 10 volts is best obtained from the lighting circuit by means of a switch-board provided with suitable shunts. The strength of the field is calculated by dividing the value of the voltage applied by the distance in cms. between the copper wire electrodes. Other details are as described for the preceding exercise. For droplets of aniline in water a velocity of about 10^{-3} cms. per second is usually observed in a field of strength 1 volt per cm.

EXERCISE 54.

Electric Endosmose through a Porcelain Cell.

The apparatus is represented in fig. 19.[1] The porcelain cell A —a filter—is supported in a stand so that it hangs freely in the large beaker B. It is closed by the doubly-bored rubber stopper 1. Through one hole passes the glass tube 2, which is bent as shown at its lower end, and is filled with a 3 per cent. jelly of agar made up in saturated KCl solution (p. 121) except at the end 3 of the short limb, which is left empty in order to retain any of the strong KCl solution that might tend to diffuse out. The limb outside the cell is connected by means of rubber tube 4 to a small funnel 5. This rubber tube and funnel are filled with saturated KCl solution. The cell and the beaker are both filled with fluid of identical composition, which may conveniently be N/1000 KCl solution.

The second hole of the stopper carries the glass tube 6, which is connected by means of rubber tube 7 to a manometer tube 8, for

[1] Modified from the arrangements described by the older physicists (Wiedemann, Quincke and others.)

which a graduated 1 c.c. pipette may be used. The stopper is eased while tubes 6, 7 and 8 are sucked full of fluid; it is then driven home. On the other side the agar tube 9 is immersed in the fluid in the beaker; it has the same form as 2, and similarly carries a small funnel 10 filled with saturated KCl solution. Into each of the funnels 5 and 10 dip similar electrodes consisting of syphon tubes of KCl-agar communicating with flasks of 10 per cent. copper sulphate solution 12 into which the actual copper electrodes 13 are plunged.

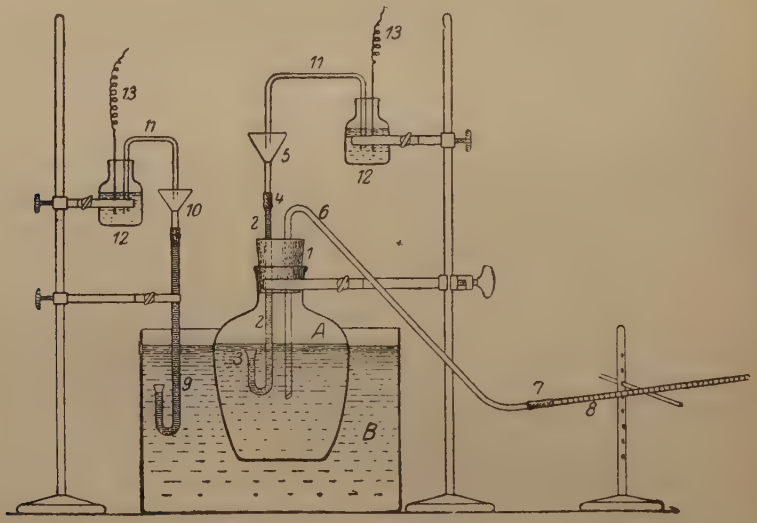

FIG. 19.—Electro-endosmose.

The tube 8 is slightly tilted at an angle of about 5–10° so that the fluid stands at a definite mark. It is necessary to be sure that the meniscus moves freely in the tube when the latter is tilted, and that it maintains its original level when left to itself. If this is so, a direct E.M.F. of 110 or 220 volts can be applied, when a movement of the water towards the cathode will be observed, no matter in which direction the current passes through the apparatus. The porcelain cell is therefore negatively charged with respect to the solution. The rate of transfer of the water, as shown by the creeping of the meniscus in the pipette, is of the order of ·1–·2 c.c. per minute.

EXERCISE 55.

Electric Endosmose through a Collodion Membrane.[1]

The arrangement is the same as that of the previous exercise except that the porous cell is replaced by a collodion membrane which is conveniently made as follows: A cylindrical glass tube of 15 cm. height and 3 cm. diameter is expanded at one end into a rim 3 mm. in width, which must be ground flat. A circle of filter paper soaked in collodion is pressed on to this rim, and bent round at the edges to be stuck with the aid of a further supply of collodion to the sloping glass surface. The filter paper is now treated inside and out with a further thin layer of collodion, and then dried. After the tightness of the junction has been tested the cylinder is used instead of the porous filter in the previously described apparatus. The result is similar, but the rate of movement of the water is slower. Through a collodion membrane the movement is always under all circumstances towards the cathode. If instead of the N/1000 KCl solution a strongly acid solution ($^1/_{100}$ N. HCl), or one of $AlCl_3$ is used the movement of the water may become very sluggish, or even be completely arrested, but it never becomes reversed.

If the collodion membrane is soaked for several hours in a 1 per cent. solution of gelatin it takes on the properties of a gelatin membrane,[2] that is, in acid solutions it becomes positively charged with respect to the solution, while in alkaline solutions the sign of its charge is reversed. $Al^{\cdots}$ ions will also produce a reversal of the charge.

Membranes of gelatin and of agar can also be made without a substrate of collodion by sticking the filter paper to the glass cylinder with a fluid solution of these substances and then treating it with more of the same solution. The pure gelatin membrane shows the same amphoteric behaviour as the gelatinised collodion; but agar, like the pure collodion, invariably carries a negative charge.

[1] Modified from Jacques Loeb, *Journ. Gen. Physiol.* 1, 1918.
[2] Jacques Loeb, l.c.

X.

Adsorption.

EXERCISE 56.

A Survey of the various Types of Adsorbents and Adsorbable Substances.[1]

There are various types of adsorbents:

1. Charcoal. Of all known adsorbents this has the most powerful and most varied activity, adsorbing electrolytes and non-electrolytes, acid and basic dyes, and, to a particularly marked extent, surface-active non-electrolytes.

The most suitable form in which to employ it is "Carbo animalis" Merck. This preparation is not quite free from ash, and its moisture content amounts, under ordinary circumstances, to something like 20 per cent., but this can be neglected in the experiments that follow.

2. Insoluble powders possessing the characters of electrolytes (insoluble salts, acids and bases). Their charge (with respect to water) is usually negative as in the case of kaolin, for example, but in rare instances is positive, as in the case of ferric hydroxide.

3. "Indifferent" organic substances of the nature of cellulose, for example, filter paper and cotton wool, which carry a negative charge.

4. Amphoteric organic substances of the protein type—silk, wool, fixed tissue proteins.

Of the materials to be adsorbed the following solutions are made up:

1. A saturated aqueous solution (see p. 83) of tri-butyrin or heptyl- or octyl-alcohol as a type of a sparingly soluble substance with a powerful surface activity.

2. ·1 N. acetic acid as a type of a weak electrolyte with a distinct but not excessive degree of surface-activity.

3. An approximately ·1 N. (·58 per cent.) solution of acetone as an example of a non-electrolyte possessing the same degree of surface-activity as acetic acid.

[1] Taken from the experiments of H. Freundlich, *Zeitschr. f. physik Chemie*. and L. Michaelis and P. Rona, several papers, in *Biochem. Zeitschr.* for 1908, 1919 and 1920.

4. A 2 per cent. solution of glucose as an example of a non-electrolyte that does not diminish but, on the contrary, slightly increases the surface-tension.

5. A 1 in 10,000 solution of eosin which is a non-colloidal acid dye.

6. A 1 in 10,000 solution of the colloidal acid dye Congo red.

7. A 1 in 10,000 solution of methylene blue as an example of a basic dye.

The exact concentration of each solution is first determined by analysis; 100 c.c. of it are then mixed with 2 gms. of charcoal, or 20 gms. of kaolin, or 20 gms. of ferric hydroxide, as the case may be, the mixture is shaken in a flask for 3 minutes and then filtered. The concentration of the filtrate is also determined.

The acetic acid is estimated by titration against ·1 N. caustic soda using phenolphthalein as indicator; acetone is estimated by titration against standard iodine, and glucose by means of the polarimeter.

The adsorption of tri-butyrin (or heptyl- or octyl-alcohol) is followed by determining the drop number of the original solution and that of the filtrate after adsorption. In the case of the dyestuffs the depth of colour is a sufficient indication of the concentration.

In the case of charcoal it will be found that:

Tri-butyrin (or heptyl- or octyl-alcohol) is completely adsorbed; the filtrate is found to have the same drop number as pure water.

Acetic acid and acetone are adsorbed to an easily recognisable extent.

The degree of adsorption of glucose is small but recognisable; it may be made more evident by using 3–4 times as much charcoal as recommended above.

All the dye-stuffs are completely adsorbed.

In the case of kaolin neither tri-butyrin (nor the higher alcohols) nor acetone nor glucose is adsorbed, so that even a substance possessing a high degree of surface activity is not necessarily adsorbed to an appreciable extent by kaolin. Methylene blue and Congo red are adsorbed, but not eosin, so that kaolin adsorbs basic dyes and also such acid dyes as are in colloidal solution. The behaviour of ferric hydroxide is similar except that it adsorbs acid dyes, but not basic substances such as methylene blue.

From these results it will be seen that the adsorption of the

so-called surface-active substances is determined by the chemical nature of the surface to which they are exposed, and not merely by its extent. Many powders that possess the greatest conceivable specific surface (kaolin and ferric hydroxide are among them) will not adsorb a trace of a surface active non-electrolyte.

EXERCISE 57.

The Phenomenon of Adsorption-Displacement.

Two adsorbable substances occurring together in solution will mutually displace each other from an adsorbing surface. If the one is only sparingly, and the second powerfully, adsorbed the latter may expel the former completely from the surface.

1. (a) 100 c.c. of ·1 N. acetic acid + 5 c.c. of water + 2 gms. of charcoal. The filtrate will be found to contain less acetic acid than the original solution.

   (b) 100 c.c. of ·1 N. acetic acid + 5 c.c. of acetone + 2 gms. of charcoal. The amount of acetic acid adsorbed from this mixture is not so great as in (a).

Also:

1. (a) 100 c.c. of 2 per cent. glucose solution + 5 c.c. of water + 5 gms. of charcoal. The filtrate shows a considerably diminished concentration of sugar.

   (b) 100 c.c. of 2 per cent. glucose solution + 5 c.c. of acetone + 5 gms. of charcoal. The filtrate has the same concentration of sugar as the original solution.

The two (b) experiments should be carried out twice, bringing first into contact with the charcoal in the one case the one, and in the second case the other, of the adsorbable substances. In each case the result will be the same; a true reversible condition of adsorption equilibrium is attained.

EXERCISE 58.

Adsorption of Electrolytes and of Dye-stuffs.

The adsorbability of an electrolyte is determined additively by the adsorbabilities of its separate ions. Among the ions that occur under physiological conditions the most powerfully adsorbed are those of $H^{\cdot}$ and OH'. For this reason HCl and NaOH are more strongly adsorbed than NaCl.

100 c.c. portions of ·1 molar solutions of each of these three substances are treated with 15 gms. of charcoal. In the case of NaCl the adsorption is only just recognisable by titration with silver nitrate; in the cases of HCl and NaOH it is practically complete.

The adsorption of HCl is promoted by the presence of NaCl—a phenomenon that forms a counterpart of that of adsorption displacement.

100 c.c. of ·01 N. HCl + 1 gm. charcoal. The filtrate will be found to have a concentration of about ·006 N. HCl.

100 c.c. of a ·01 N. solution of HCl in approximately N. KCl, i.e. a mixture of 1 c.c. of 1 N. HCl + 99 c.c. N. KCl + 1 gm. of charcoal. The HCl concentration of the filtrate will be found to be about ·0045 N.

Adsorption on to filter paper (cellulose) is most easily investigated by the method of "capillarisation." Strips of filter paper 1 cm. wide and 20 cms. long are dipped into 1 per cent. solutions of methylene blue, eosin and Congo red. Methylene blue is a basic dye; it is strongly adsorbed so that it does not creep far up the paper, and only pure water is left to reach the higher levels. Eosin is an acid dye that exists in true solution. It is but feebly adsorbed and so climbs to practically the same height as does the water. Congo red is also an acid dye, but is a colloid; it is adsorbed in the same way as methylene blue. It is very probable that the adsorbing power of cellulose is bound up with the presence of the mineral substances and in particular with the quantities of calcium silicate that it invariably contains.

If the strips are dipped into a solution of eosinate of methylene blue (a few drops of May-Grünwald stain added to 10 c.c. of distilled water) the dyes become separated, the eosin rising to a higher level than the methylene blue. If, however, the mixture is diluted with chloroform instead of water the methylene blue will be observed to rise higher than the eosin.

A 1 per cent. aqueous solution of eosin is treated with a little dilute hydrochloric acid and then 10 c.c. of the acidified mixture are shaken with an equal volume of xylol. When the xylol has settled it is seen to be colourless, but if a strip of filter paper is immersed in the colourless solution it becomes stained intensely red.

A similar experiment with a basic dye is the following:

A 1 per cent. aqueous solution of Nile-blue is treated with a little

NaOH and the mixture is shaken with xylol as before. A brownish-red xylol layer is obtained, and if a strip of filter paper or a piece of wool be immersed in this it is at once stained a deep blue.

If a blood smear prepared on a slide by the ordinary clinical hæmatological technique is fixed for $^1/_4$ of an hour in alcohol (or for 2 minutes in methyl alcohol) and then stained in a dilute aqueous solution of eosin (1 in 1000, the addition of a trace of acetic acid is advantageous) the red corpuscles and the protoplasm of the polynuclear leucocytes take up the stain, but the nuclei remain uncoloured. If, on the contrary, the smear is stained with a basic dye (methylene blue or Nile-blue, 1 in 1000) the structures which stain most deeply are the nuclei of the polynuclear leucocytes, and with a suitable strength of stain it is practically only these that take up any colour at all.

If, however, we stain such a preparation with the solution in xylol of the eosin acid or of the Nile-blue base the plasma proteins show as a diffusely stained background, but the nuclei show not a trace of colouration with the Nile-blue. The theoretical meaning of these phenomena, so important in histological studies, is as yet unknown. They are introduced here in order to stimulate further investigation.

Another example is furnished by the staining of a dry smear of bacteria. A loop-full of B. coli is added to a drop of water on a slide and then spread out and allowed to dry in the cold. After being fixed in the ordinary way in the flame it is stained for several minutes in a 1 per cent. solution of methylene blue. On washing with water it is found that the stain is fast, that is that it cannot be dissolved out. In this case the adsorption is irreversible. But if the preparation is counterstained with a 1 per cent. solution of either Bismarck brown or chrysoidin (which, like methylene blue, are both basic stains) the methylene blue is extracted and replaced by the second dye.

The following experiment is somewhat analogous. A solution of invertase is prepared by diluting 4-fold the stock solution made according to the directions given on page 192, and to this is added an equal volume of a 4-fold dilution of the stock solution of ferric hydroxide. A thick precipitate is produced that carries down with it the whole of the ferment so that the filtrate may be shown by polarimetric observations to be without action on cane-sugar. Further, the precipitate will not give up the enzyme when shaken

with water. But if the precipitate is treated with a 5 per cent. solution of cane sugar the latter is inverted, and if the mixture be filtered after half-an-hour's gentle shaking the inversion still continues in the filtrate. The enzyme has therefore been dissolved out by the sugar.[1]

In the case of the methylene blue and chrysoidin the second dye has taken the place of the first; in the case of the ferment the mechanism of the leaching out by the sugar is not yet explained.

EXERCISE 59.

The Freundlich Adsorption Isotherm.

If the volume and temperature of a solution are kept constant while the quantities of adsorbent and of material to be adsorbed are varied it is found that when adsorption equilibrium has been established the relation between the amount of the substance adsorbed (x) and the concentration of that remaining in the solution (c) is given by the equation:

$$\frac{x}{m} = k \cdot c^n,$$

where m is the weight of adsorbent present and k is a constant depending on the nature of the particular adsorbent used and furnishing a measure of the adsorbing power when determined for different adsorbents by allowing them to act on the same adsorbable material. n is a constant whose value is to a certain extent dependent on the nature of the substance undergoing adsorption, but is not usually very far removed from ·5. Acetic acid and acetone are recommended as suitable materials for the confirmation of this formula. 100 c.c. of each of a number of solutions of the selected substance varying in strength from ·3 to ·002 normal are placed in well-stoppered flasks containing already previously exactly weighed out quantities of about 1 gm. of charcoal. After 5 minutes' shaking the contents of the flasks are filtered, the first portion of each filtrate rejected, and a later portion used for the titration. The initial solutions should also be standardised by titration. The total concentration of the substance in 100 c.c. of the solution before treatment with the charcoal and the total amount of it remaining in the 100 c.c. after the adsorption are calculated from the results of the titrations.

[1] A. Eriksson, *Z. f. physiol Chemie*, **72**, 313, 1911, and O. Meyerhof, *Pflüger's Archiv*, 157, 251, 1914.

These quantities are best expressed in millimols. (1 millimol of acetic acid is the amount corresponding to 1 c.c. of N. NaOH; 1 millimol of acetone = ·058 gm.)

The following is an example of the type of result to be expected:

Millimols of acetone in 100 c.c. of the orig. solution. a	Weight of charcoal. (gms.) m	Concentration in the filtrate		Quantity adsorbed	
		Millimols per 100 c.c. F	In terms of normal. c	Total. $x=a-F$	per gm. charcoal. x/m
·421	·8987	·234	·0234	·187	·208
2·103	1·0320	1·465	·1465	·638	·618
5·257	1·0688	4·103	·4103	1·154	1·077
10·50	1·0951	8·862	·8862	1·64	1·498
20·34	1·2425	17·76	1·776	2·58	2·08
30·52	1·2556	26·90	2·690	3·62	2·88

In order to evaluate k and c we proceed as follows:

Taking logarithms of both sides of the equation on page 135:

$$\log. \frac{x}{m} = \log. k + n \cdot \log. c$$

$\log. \frac{x}{m}$ is thus a linear function of log. c and n is the tangent of the angle between the straight lines expressing these quantities. The significance of k is seen as follows:

$$\log. k = \log. \frac{x}{m} - n \log. c.$$

When $c=1$, $n \log. c=0$ and $\log. k = \log. \frac{x}{m}$.

We collect first of all the corresponding values of $\log. \frac{x}{m}$ and $\log. c$:

$\log. \frac{x}{m}$	$\log. c$
−0·682	−1·631
−0·209	−0·834
+0·032	−0·387
+0·176	−0·052
+0·318	+0·247
+0·459	+0·430

When these values are plotted against each other the diagram in fig. 20 is obtained.

From this by graphic measurement it is found that $n = \cdot 52$; log. $k = \cdot 75$, so that $k = 5 \cdot 62$.

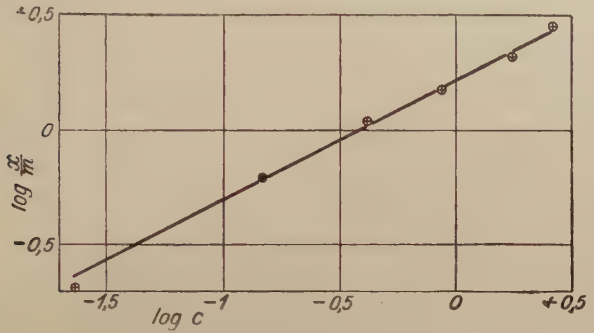

FIG. 20.—Adsorption Isotherm for Acetone.

The actual value of k depends on the units of concentration used, and will differ very considerably according as to whether the concentrations are expressed in gm.-mols. per litre, or, as here, as millimols per 100 c.c.

The value of n, on the contrary, is independent of the units chosen. The straight line in the figure is drawn so by eye so as to lie as evenly as possible between the observed points, the lowest point of all being neglected. The marked divergence of this point from the line is not due to experimental error, but is an expression of the circumstance that the Freundlich adsorption isotherm is only an approximate expression of the relationships, and can be used as an interpolation formula over only a restricted range.

EXERCISE 60.

The Oxidation of Oxalic Acid on the Surface of Charcoal.[1]

The velocities of many chemical reactions are considerably accelerated when the reacting substances are present in a condensed condition on an adsorbing surface. The surface thus acts as a catalyst. This phenomenon is of physiological importance, because there is a group of ferments whose catalytic activity seems to depend on such a process of surface condensation. According to O. Warburg this seems to be the case with the enzymes concerned with respiration, their activity being dependent on the maintenance of the

[1] From O. Warburg, *Pflüger's Archiv.* **155**, 547, 1914.

normal cell structure, diminishing when the degree of dispersion of the cell substance is diminished and being completely inhibited by surface-active substances that expel the reacting materials from the adsorbing surfaces. Charcoal serves as a model of such a respiratory ferment. Many substances that are stable towards atmospheric oxygen, such as oxalic acid and amino-acids, are oxidised when condensed on the surface of this adsorbent.

The oxidation of oxalic acid can be demonstrated in a qualitative manner as follows: One limb of a glass T-piece is connected with a water pump, the second limb is fitted with a short length of rubber tubing provided with a screw clip which serves to regulate the size of the aperture, and so the effective rate of suction, while the third limb of the T-piece is connected to a series of flasks of which we will regard the one furthest from the pump as No. 1. This first member of the series is, as a matter of fact, a soda-lime tower of the form shown in fig. 1, page 34, members 2 and 3 are gas wash-bottles filled with a deep layer of strong caustic soda solution; No. 4 is a wash-bottle filled with baryta solution; No. 5 is the actual reaction vessel and contains at first a suspension of 5 gms. of Merck's blood charcoal in 100 c.c. of distilled water. In order to expel any adsorbed CO_2 this suspension must be boiled for 15 minutes and run into the reaction vessel while still warm. The reaction vessel is warmed throughout the experiment by immersion in a dish of hot water. No. 6 is again a gas wash-bottle containing baryta solution; it stands next to the pump. By adjusting the clip on the T-piece and also a similarly arranged one on the distal end of the soda-lime tower the air stream is regulated so that the individual bubbles follow one another at a moderate rate. Flasks 1 to 3 serve to adsorb the CO_2 from the entering air, and the baryta flask No. 4 serves to indicate that this adsorption is complete. The final baryta flask serves to indicate the time at which the charcoal suspension ceases to evolve CO_2, for even after the boiling the material continues to evolve CO_2 which may either exist pre-formed or be produced by the catalytic oxidation of organic substances on the charcoal surface. As soon as the baryta in flask 6 begins to show a turbidity it is disconnected from the series and then a stream of air is maintained through the apparatus for about an hour or so, the reaction flask No. 5 being kept warm meanwhile in hot water. A freshly filled baryta flask is now placed in position No. 6, and if this shows no turbidity on passing the air current for a further period of 5–10 minutes, 2 gm.

of oxalic acid are added to the reaction flask No. 5, which is then at once closed and maintained in its water bath at 40–45° C. If the air current is now again turned on, the baryta solution in flask 6 shows a well-marked turbidity in the course of 10–15 minutes. The oxalic acid is thus burnt or " respired " on the surface of the charcoal.

The success of the experiment depends on the kind of charcoal used. According to O. Warburg to oxidation is facilitated by even minimal traces of iron.

XI.

The Influence of Hydrogen Ion Concentration on the Activity of Enzymes.

Not only on the colour of indicators in a state of molecular dispersion (to take but one example among reactions as occurring in true solution), and not only on the condition of colloids, but also on the activities of enzymes is the influence of hydrogen ions so powerfully pronounced that the effects of all other ions that occur under physiological conditions in comparison amount to a mere correction term. There are, of course, exceptions to this statement; for example, the action of the salivary diastase, ptyalin, is very powerfully influenced by the prevailing Cl′-ion concentration; but, even so, the above law is found to apply if we regard the compound of the ferment with the Cl′-ion as the true active material.

The explanation of this action of the hydrogen ion may be given from two points of view; one can either ascribe the effect to the action of the hydrogen ion on the ionisation of the enzyme considered as an electrolyte, or can regard it as due to the influence of the hydrogen-ion on the colloidal condition of the ferment. Both explanations are possible; they are not mutually exclusive, and between the two extremes they represent many transitional cases occur. In this way we can account for the behaviour of enzymes that exist in a condition approaching molecular dispersity (invertase) as well as of those that are highly colloidal in nature (zymase, respiratory enzymes).

EXERCISE 61.

The Influence of Hydrogen Ion Concentration on the Activity of Ptyalin.[1]

The activity of ptyalin in hydrolysing starch is very much influenced by the concentration of the Cl′ and H·-ions. The enzyme has no activity at all unless Cl′ or some similar ion such as Br′ is

[1] Quoted from W. E. Ringer, *Zeitschr. f. physiol. Chemie.* **67**, 332, 1910; L. Michaelis and H. Pechstein, *Biochem. Zeitschr.* **59**, 77, 1914.

present. As the concentration of Cl′-ions is increased the activity is also increased up to a certain maximum that is not exceeded on further increase of the Cl′-ion concentration. A concentration of this ion of a few tenths per cent. is sufficient to develop this maximum activity. In the case of the H·-ion the relationships are different. At a certain value of h there exists an optimum activity which is diminished at hydrogen ion concentrations either greater or less than this most favourable value. In the following experiment we shall maintain throughout a constant and sufficient concentration of Cl′-ion and vary the reaction in order to determine the optimum value of h.

2·5 gm. of soluble starch is boiled in 500 c.c. of ·3 per cent. NaCl solution. A 50 c.c. portion of the resulting liquid is placed in each of 7 Erlenmeyer flasks. The following additions of phosphate solutions prepared as recommended on page 85 are then made:

No.	1	2	3	4	5	6	7
M/3 primary phosphate c.c.	4·7	4·4	3·3	2·5	1·7	·6	·3
M/3 secondary phosphate c.c.	·3	·6	1·7	2·5	3·3	4·4	4·7

A series of about 30 test-tubes, each containing 5 c.c. of very dilute (about N/1000) iodine solution, are now placed in readiness. To each Erlenmeyer flask in turn is now added 5 c.c. of a 100- to 1000-fold dilution of saliva, an interval of exactly 2 minutes being allowed to elapse between the additions to the successive flasks. Then at 2-minute intervals 5 c.c. of the contents of flask No. 4 are removed and run into an iodine tube. At first a blue colouration will be observed, later a violet colour, and finally red. When the colouration produced is intermediate between reddish-violet and pure red the final series showing the relative rates of the reaction in the successive flasks can be set up. From each flask in turn, and with an interval of exactly 2 minutes between the one flask and the next, 5 c.c. of the reaction mixture are withdrawn and added to separate tubes of iodine. The resulting colourations will be somewhat as follows:

1	2	3	4	5	6	7
blue	violet	red	yellow-red	red	red-violet	violet

The action of the ferment has proceeded farthest in solution No. 4. If the pH of this solution be measured according to the method given on page 52 it will be found to be about 6·8.

EXERCISE 62.

The Optimum Reaction for the Activity of Pepsin.[1]

The activity of pepsin is so powerfully influenced by changes of hydrogen ion concentration that the effects of other ions are vanishingly small in comparison. This is especially true when the protein to be digested is in solution and not in the solid or coagulated form.

Two gms. of edestine are soaked for 24 hours at 37° C. in 50 c.c. of ·1 N. NaOH; the solution is then diluted to 490 c.c. with water, and finally to 500 c.c. with 1 N. HCl. There is thus obtained a ·4 per cent. solution of the protein in ·01 N. HCl + ·01 N. NaCl which we will designate just simply as "edestine solution."

A ·5 per cent. solution of Grübler's pepsin is also prepared; in what follows this is referred to as the "pepsin solution." A preliminary experiment is first carried out by placing a mixture of 6 c.c. of edestine solution + ·4 c.c. of 1 N. HCl + ·5 c.c. of pepsin solution in each of a series of 5–6 test-tubes kept at room temperature. At intervals of several minutes the digestion is interrupted in each tube in turn by the addition of an abundance of solid sodium acetate which precipitates the edestine that still remains unchanged. In this way one determines roughly how many minutes are required for the digestion of a considerable part, but not of the whole of the protein. Once this time interval has been determined the following series can be set up:

No.	1	2	3	4	5	6
Edestine solution c.c.	6	6	6	6	6	6
1 N. HCl c.c.	0	·2	·4	·8	1·6	3·2
Distilled water c.c.	4	3·8	3·6	3·2	2·4	·8
Pepsin solution c.c.	·5	·5	·5	·5	·5	·5

After the lapse of the time interval determined in the preliminary experiment the digestion is brought to an end in all the tubes by the addition of solid sodium acetate. It will be found that the digestion has progressed most rapidly in tube No. 2. The measurement of the pH of the contents of this tube must be made on another portion of a similar solution before the addition of the sodium acetate. The electrometric method is the most suitable in this case. The optimum will be found to be at a pH of about 1·7.

[1] L. Michaelis and A. Mendelssohn, *Biochem. Zeitschr.* **65**, 1, 1914.

EXERCISE 63.

The Optimum Reaction for the Activity of Catalase.[1]

A strongly active preparation of catalase can be obtained as follows[2]: 50 gm. of calves' liver is ground up with sand and kieselguhr in a mortar, and stirred up with 30 c.c. of 93 per cent. alcohol. After a quarter of an hour the pulp is well pressed and the press-juice rejected. The press-cake is now well stirred up with water and again pressed, and then finally washed once more with water in the same way. The aqueous press-juices are to be united and preserved; they constitute the stock catalase solution. This will keep for a considerable time if a little toluene is added to it. The precipitate that slowly forms and settles is to be filtered off before use. For our purposes we shall require a litre of a 50,000-fold dilution of this ferment and also about a litre of hydrogen peroxide solution obtained by diluting Merck's perhydrol 1000-fold.

The following mixtures are made up in a series of conical flasks:

No.	2	3	4	5	6	7
·1 N. sodium acetate c.c. .	2	2	2	2	2	2
·1 N. acetic acid c.c. .	0	·12	·5	2	—	—
1 N. acetic acid c.c. .	—	—	—	—	·8	3·2
Distilled water c.c. . .	3·2	3·08	2·7	1·2	2·4	0

The flask which occupies position 1 contains 1 c.c. of M/15 secondary sodium phosphate (see p. 34) + 4·2 c.c. of distilled water. To each flask in turn is now added 100 c.c. of the hydrogen peroxide solution, and then at intervals of 2 minutes between successive flasks 50 c.c. of the diluted ferment solution. From flask No. 2 a test portion of 10 c.c. is withdrawn every 5 minutes, treated with about 5 c.c. of dilute sulphuric acid and then titrated with N/20 permanganate. At first about 3 c.c. of permanganate will be required, but after 10–20 minutes only about half this quantity. When the suitable progress of the reaction has thus been confirmed, a portion of 25 c.c. is withdrawn from each flask in turn, the usual 2 minutes' interval being allowed between one flask and the next, and poured into a vessel containing 10 c.c. of dilute sulphuric acid and titrated with the permanganate. The titrations need not be carried out at once after the mixing with the sulphuric acid, but

[1] L. Michaelis and H. Pechstein, *Biochem. Zeitschr.* **53**, 320 (1913). S. P. L. Sörensen, *Biochem. Zeitschr.* **21**, 131 (1909).

can be postponed until all the test samples have been withdrawn. It will be found that each flask requires less permanganate than corresponds to the amount of hydrogen peroxide originally present. The amount of decomposition is greatest and about equal in solutions 1 to 3; it then becomes smaller in the remaining members of the series until No. 7 requires almost the initial quantity of permanganate. The optimum rate therefore occurs over a wide range of reaction from strongly alkaline (pH = almost 9) to pH = 5·5. In this experiment the optimum reaction is exceeded only on the acid and not on the alkaline side. The pH values can be determined colorimetrically in the remaining portions of the mixtures; preferably after all the hydrogen peroxide has decomposed.

XII.

Measurement of the Electrical Conductivity of Solutions.

EXERCISE 64.

Figure 21 gives a schematic representation of the apparatus as arranged by Kohlrausch. A is an accumulator which is connected through a switch and a sliding adjustable resistance with the primary of a small inductorium I. The total value of the adjustable resistance need amount to only a few ohms, and the effective value is so adjusted that the contact-breaker of the induction coil is just kept in continuous vibration. The weaker the current that can be used the better. The terminals of the secondary coil are connected up as shown. R is a resistance-box with a range of at least from 1 to 1000 ohms; for feebly conducting liquids a range from up to 10,000 ohms will be required. W is the resistance to be measured consisting of an electrolytic cell that is described in detail below. *ab* is a thin wire 1 metre long of platinum-iridium or of constantan stretched alongside a scale of millimeters. *d* is a sliding contact and T is a telephone. The inductorium should be covered with a box so that its own note is not directly audible.

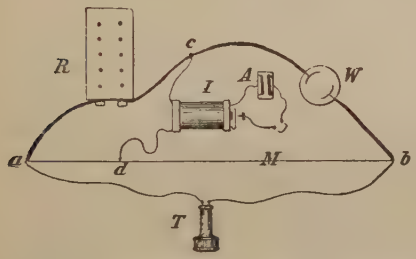

FIG. 21.—Scheme of the Apparatus for the measurement of Electrical Conductivity.

When the ratio of the resistances *ad* and *db* is equal to the ratio of the resistances R and W the potential difference between the points *a* and *b* is zero and the telephone becomes silent. The essential process in the measurement of the unknown resistance W consists, therefore, in the determination of the position of the sliding contact at which the sound emitted by the telephone is a minimum.

The value of R may be chosen at will; but it is better that it should be of such a magnitude that the point at which the telephone is silent is not far removed from the mid-point of the scale; the experimental error is then a minimum.

It is assumed that both the resistance box and the slide-wire have been previously calibrated and that the necessary corrections are known. (See Ostwald-Luther: Physiko-chemische Messungen.)

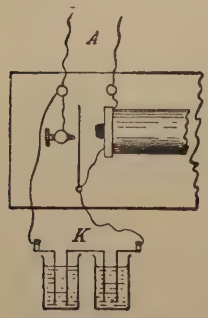

FIG. 22.—Arrangement of the Condensor K for reducing sparking at the contact breaker.

It is an advantage to minimise the sparking at the contact-breaker of the inductorium by connecting in parallel with the contact points a small condenser of $^1/_2$ micro-farad capacity, as in fig. 22 (K).

The point of minimum tone is best located exactly by sliding the contact *d* up and down over a certain length of the wire in the neighbourhood of the correct point while the telephone is held continuously at the ear; the range of the movement is then gradually reduced until the position of balance can be defined to within 1 mm. The sharpness of the point of minimum sound is greater the surface of the electrodes in the resistance cell, and also the greater, up to a certain limit, the value of the resistance to be measured. The surface of the electrodes is increased by coating them with platinum black.

The resistance cell most suitable for physiological purposes is the form devised by Arrhenius and shown in the accompanying figure 23. The electrodes consist of two platinum plates, stout enough to be inflexible, mounted rigidly and immovably on short thick platinum wires that are fused through glass tubes and serve to make communication with the contained mercury into which the wires from the external circuit dip. The plates are first cleaned with bichromate-sulphuric acid mixture, well rinsed with water and then platinised by being made to function as electrodes in a vessel of Lummer's solution (1 gm. platinum chloride + ·02 gm. of lead acetate dissolved in 100 c.c. of water). Current is supplied from a 4-volt accumulator

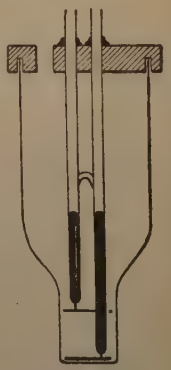
FIG. 23. Conductivity Cell.

and is reversed at intervals during a period of 10–15 minutes. At the end of this time the electrodes should be uniformly black. Electrodes that have been platinised previously require only 1–2 minutes treatment. The plates are now washed in water and then, in order to reduce the last obstinately adhering traces of platinum salt, are used as electrodes in a vessel of dilute sulphuric acid, the current through which is reversed repeatedly. The electrodes are finally washed for several hours in distilled water, which must be frequently changed.

Seeing that the conductivity is very strongly influenced by temperature changes, the conductivity cell must be kept during the measurement in a water bath whose temperature is exactly regulated.

The experiment is begun with the measurement of the conductivity of a very carefully prepared ·1 N. solution of KCl. The salt must be of the purest quality (e.g. Kahlbaum's "for analysis") and should be brought to a dull red heat and then cooled in the desiccator before being weighed out. 7·44 gms. of it are to be dissolved and made up to a litre with water that has been previously boiled out—or preferably with conductivity water (see below). If the resistance is found to be W ohms then we say that the conductivity of the KCl solution in this particular conductivity cell is $\frac{1}{W}$. But the conductivity must be expressed in terms of a layer of solution 1 cm. thick and with a cross-section of 1 cm.^{2}; the conductivity of such a mass of solution is termed the specific conductivity of the solution, K. The specific conductivity of ·1 N. KCl solution is known and amounts

at 18° C. to ·01119
,, 19° C. ,, ·01143
,, 25° C. ,, ·01288
,, 35° C. ,, ·01539.

If, therefore, a conductivity of $\frac{1}{W}$ has been found in the vessel used, this must be multiplied by the factor $W \times ·01119$ (at 18° C.) in order to give the specific conductivity, and every measurement made with this vessel must be similarly multiplied with this same factor in order to convert it into specific conductivity. This factor which we will denote by C is therefore characteristic of the particular conductivity cell for which it has been determined; it is referred to

as the capacity of the cell. W is, as we have mentioned, the resistance of the ·1 N. KCl solution in the cell, measured in ohms; its value should be frequently checked, particularly after each replatinisation of the electrodes. To take an example, suppose that W was found to be 50 ohms, then the capacity of the vessel is $C = ·01119 \times 50 = ·5595$. If now an unknown fluid in the same vessel showed a resistance of 60 ohms the conductivity of this solution in this vessel is $\frac{1}{60}$, so that the specific conductivity

$$K = \frac{1}{60} \times ·5595 = ·00933.$$

If the fluid under investigation is a solution of a single pure substance of molecular concentration c, then $\frac{K}{c}$ is the molecular conductivity μ.

If the solution has a very small specific conductivity (less than about ·001) it is not sufficient to purify the water used as solvent merely by expelling the dissolved CO_2 by boiling, because other impurities that may be present may produce appreciable errors. " Conductivity water " must be used in such a case. Further, it is advisable also to use blank unplatinised electrodes.

The specific conductivity of the purest water known has a value of $·4 \times 10^{-7}$. Water that has been twice distilled and condensed in silver condensers has a conductivity usually not exceeding 2×10^{-6} and is sufficiently pure for most purposes. Ordinary distilled water has a conductivity according to its CO_2 content of about 10^{-5}. Conductivity water can be obtained in paraffined glass bottles from Kahlbaum; care must be taken to protect it from the atmospheric CO_2 while it is being withdrawn for use.

These instructions will suffice for practice in the measurement of the conductivity of blood, urine, etc. For other purposes see Ostwald-Luther: *Physiko-chemische Messungen.*

In a laboratory where the apparatus described in the succeeding sections for the measurement of E.M.F.'s is set up this may also be used for the measurement of electrical conductivities if a double rotating commutator of the pattern described by Whetham *Phil. Trans. Roy. Soc.*, London, 194A, 321, 1900, is added to the equipment. The alternating current from the commutator is passed through a circuit comprising the conductivity cell, a standard

resistance box and an adjustable resistance in series. The E.M.F. across the standard resistance and that across the conductivity cell are measured by means of the potentiometric arrangement after having been rectified by means of the commutator. Seeing that the same current passes through each portion of the circuit a simple application of Ohm's law shows that the ratio of the unknown to the standard resistance is equal to the ratio of the E.M.F.'s across these two members of the circuit. An interesting exercise is the investigation of the relation between the conductivity of blood and the concentration of its corpuscles, for which purpose it is necessary to measure the conductivity of the concentrated suspension of corpuscles obtained by centrifuging, and of several known dilutions of this with its own plasma.—*Translator.*

XIII.

Measurement of Electromotive Forces.

The measurement of electromotive forces has proved to be a method of amazing usefulness in biochemistry. In its many-sided applications and in the certainty of interpretation of its results it is far superior to the measurement of electrical conductivity. It furnishes the basis for the fundamental method of measurement of hydrogen-ion concentrations by means of which all other methods are standardised. And the range of its application is still expanding.

EXERCISE 65.

Preparation of a Standard Cell.[1]

The most useful standard of electromotive force is the cadmium standard cell. Its glass containing vessel has an H-shaped form, each limb being provided with a fused-in platinum wire which leads to the appropriate terminal. To fill the cell the following reagents are required:

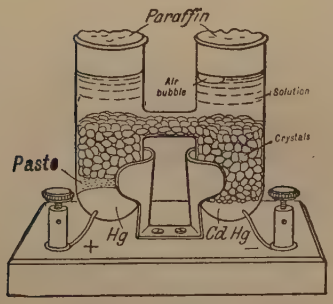

FIG. 24.—Standard Cell.

1. Pure mercury. This can be prepared from the commercial pure distilled mercury by the following method: 20 c.c. of the mercury are placed in a 50 c.c. flask, together with 20 c.c. of an approximately 1 per cent. solution of mercurous (not mercuric) nitrate and a few drops of nitric acid. After half-an-hour's vigorous shaking the aqueous layer is poured off, the mercury is washed with distilled water and is then submitted to a further treatment with a fresh portion of the same solution. When the metal has been once more thoroughly washed with distilled water it is poured into a porcelain dish, as much of the water as possible is poured off, and then the rest is sucked up with pieces of filter paper. If it is required to filter the mercury this can be done through a filter paper in which a few small holes have been pricked by means of a fine glass point.

[1] Taken in the main from Ostwald-Luther, *Physiko-chemische Messungen.*

2. Pure cadmium. This should be purchased in lumps weighing only 1-2 gms., as it is very difficult to break up. Kahlbaum's purest cadmium has up to the present been reliable in its absolute freedom from zinc. With regard to the tests for the purity of the cadmium see Ostwald-Luther: *Physiko-chemische Messungen.*

3. Pure cadmium sulphate in the solid and also in saturated solution. The preparation of the solution should be started some days in advance as the saturation proceeds but slowly.

4. Mercurous sulphate (not the mercuric salt). This must be freed from all soluble mercury salts by being washed three times by decantation with successive portions of the cadmium sulphate solution. When the washing is finished the supernatant liquid is removed as completely as may be.

Meanwhile the cadmium amalgam can be prepared. A weighed lump of the cadmium (about 2 gms.) is melted with 6–8 times its weight of the pure mercury in a porcelain dish; the mixture is allowed to cool somewhat and then poured while still liquid into one arm of the H-tube until the fused-in platinum wire is covered by a good thick layer. The amalgam solidifies in a short time; it constitutes the negative pole of the cell.

Into the other limb an equal quantity of the pure mercury is placed. The mercurous sulphate which has meanwhile been washed is now mixed while still moist with a few drops of the pure mercury and a very little of the cadmium sulphate solution in a mortar and rubbed up into a uniform grey paste. Of this a layer about 5 mm. in height is transferred to the surface of the mercury (but not to that of the amalgam). Both limbs are now loosely packed with coarse crystals of the cadmium sulphate and then filled up with the saturated solution. The openings of the limbs are closed by means of moderately tightly fitting discs of cork, which should not project above the level of the glass. In one limb, at least, an air bubble must be left in order to take up the expansion of the liquid produced by variations of temperature. Finally, the corks are fixed and sealed by means of molten paraffin or of sealing-wax; the cell is then ready for immediate use as a standard of electromotive force. The value of its electromotive force at room temperature is 1·0187 volts, and this value does not vary appreciably over the range of temperature variations liable to occur in practice. Small departures may be observed from this value on account of differences in the degree of purity of the materials used; but using pure materials these

must not exceed $\pm$ ·0002 volt. The cell may be calibrated by comparison with a tested Weston element by balancing both in turn against the E.M.F. of an accumulator by the compensation method described later. The Weston element does not contain excess of $CdSO_4$; it is filled with a solution saturated at 4° C., and its E.M.F. amounts to 1·0186 volts. at all ordinary room temperatures. A cadmium cell will last indefinitely provided that no current is taken from it, that is that it is used only in a practically compensated circuit for the purpose of calibrating the accumulator. A single brief short-circuiting of the cell may lead to a fall of several millivolts in its E.M.F., but the correct value will be re-established after a few hours. One should always work with the home-made cadmium cell, reserving the standardised Weston element for an occasional calibration.

EXERCISE 66.

The Mode of Use of the Capillary Electrometer.

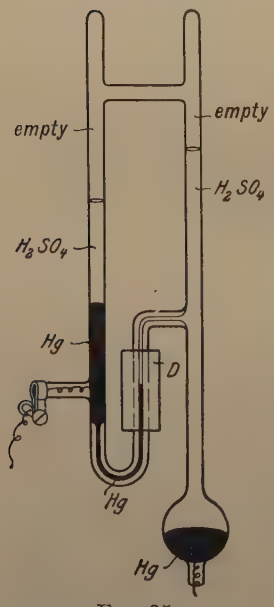

FIG. 25.
Capillary Electrometer.

The capillary electrometer consists of a special electrometer tube mounted in a stand which also carries a small microscope with a magnifying power of about 50 diameters by means of which the readings of the instrument are taken. A short-circuiting key must also be provided. The best form of electrometer tube is the closed type illustrated in fig. 25. D is a cover-glass cemented on with Canada balsam. The sensitiveness of the instrument depends on a suitable choice of diameter for the capillary. This must not be too wide, but on the other hand must not be so narrow that the free movement of the mercury is hampered. Before the apparatus is set up the tube should be tilted until the greater part of the mercury has run over into the limb that is not expanded into a bulb; the tube is now brought into an almost vertical position with the bulb side sloping only slightly downwards and mercury is allowed to drip over into the bulb until, on suddenly

righting the apparatus, the mercury stands at about the mid-point of the cover-glass. The tube can now be fixed in the stand and the microscope focussed on to the meniscus. Artificial illumination is not necessary if the table carrying the instrument is placed conveniently near the window. The leading-in wires are arranged according to the scheme in fig. 26; they must be well insulated at all points. C is the short-circuiting device; it may take the form of either a mercury dipping contact or a right-angle lever key, and is most conveniently mounted on the base of the main stand of the apparatus. The wires labelled with arrows are those that bring in the current to be measured; they must at some point include a key, which must be closed only a short time—a second or less—before the reading is to be taken. The short-circuiting key, on the other hand, must be kept continuously closed until the moment of observation, and must be closed again immediately—that is, not more than a second later.

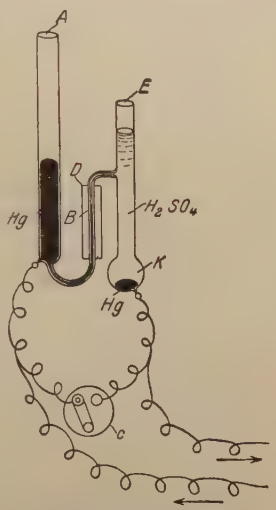

FIG. 26.—Capillary Electrometer Circuit.

After the electrometer tube has been mounted in its stand it must be left undisturbed with the short-circuit closed for several—preferably 24—hours. It is ready for use when it fulfils the following conditions:—1. When the short-circuiting key is opened with as little handling as possible the meniscus must remain at rest, or at least show no considerable movement during the course of several seconds. 2. When a weak current is passed through the tube a definite movement of the meniscus must take place. A suitable test current may be generated most simply by substituting for the copper wires denoted by the arrows in fig. 26 two short wires of different metals such as copper and brass, or two different specimens of brass, or copper and iron (or the inevitable slight difference of composition between the two terminals will often suffice), and then touching these wires with different fingers after having opened the short-circuit key. A powerful movement of the meniscus should take place, and it should occur just as easily but in the reverse direction when the wires have been interchanged. It is unnecessary for

the purpose of this test to wait for the attainment of a constant level of the meniscus; the observation of a movement through several divisions of the eyepiece scale is sufficient. The short circuit is then at once closed.

If the electrometer is insensitive its behaviour can often be improved by the following artifice. A test current, generated as above, is passed through the instrument for some minutes in such a direction that the movement of the meniscus, as seen in the microscope is upwards (the actual movement is, of course, downwards). The short-circuit key is then closed until the electrometer answers to the above-described tests. A current of long duration should never be passed through the instrument in such a direction that the apparent movement of the meniscus is downwards, otherwise a bubble of hydrogen will be formed on the meniscus, and then the apparatus has to be brought into working order from the beginning again. With appropriate treatment a single meniscus will remain in good condition for months, but a beginner usually has to renew it somewhat frequently.

EXERCISE 67.

Setting up of the Apparatus for the Measurement of Electromotive Forces by the Compensation Method using a Potentiometer Wire.

All wires used in the circuit must be well insulated throughout their entire length, and only bared to the necessary extent at their extreme ends. The contact points, particularly those of the sliding or rubbing type, as, for example, in the short circuit key of the form of electrometer we have described, should be occasionally moistened with a drop of paraffin oil.

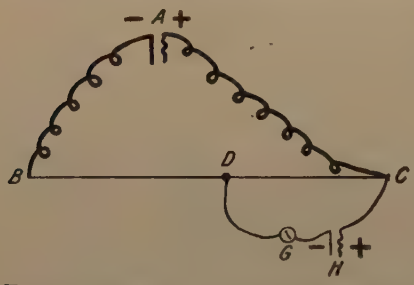

FIG. 27.—Diagram of the Compensation Circuit.

The circuit is arranged as shown in fig. 27. A is an accumulator, BC the slide wire (similar to that used in the apparatus for the measurement of electrical conductivity), D the sliding contact, G the capillary electrometer, and H the cell whose electromotive force is to be

measured—in this case the cadmium cell. Care must be taken to arrange the poles of the accumulator and of the standard element in the relative directions indicated in the figure. Otherwise the electromotive forces will not be opposed but summated, and as a result both the standard cell and the capillary electrometer will be rendered unusable for a considerable time.

A switch must be inserted somewhere between A and B and another at some point in the side circuit DGHC; these are not shown in the figure. The main circuit ABDCA is best closed some time before the beginning of the experiment and left closed during its whole duration. The wires AB and AC should be stout (1 mm. diameter) and not unnecessarily long. In the side circuit DGHCD the connecting wires may be as thin as desired.

The circuit DGHC must be left open with the capillary electrometer continuously short-circuited. The E.M.F. at H is to be supplied at first by the cadmium cell. The sliding contact should be placed at first near the mid-point of the potentiometer wire. This is the fundamental arrangement of apparatus that we shall employ throughout.

EXERCISE 68.

Measurement of the E.M.F. of the Accumulator.

The E.M.F. of the accumulator immediately after charging is well over 2 volts. But if the main circuit is kept closed for some time the E.M.F. falls a little and then remains almost constant, falling very slowly from a value of 2 volts. down to 1·85 volts. When the latter value has been reached the cell should be re-charged. It is when the accumulator is in the partially discharged condition that its E.M.F. is most nearly constant, and it is during this period that the cell is most suitable for use. Its E.M.F. is measured by means of the above arrangement in the following way:—1. Close the main circuit. 2. Open the short-circuit of the electrometer and make sure as quickly as possible that the meniscus does not shift appreciably during the course of 1–2 seconds; then close the side circuit for a fraction of a second while still observing the electrometer. As soon as the movement is evident at once open the side circuit. 3. Short-circuit the electrometer.

It will have been noticed whether the meniscus moved upwards or downwards. The slider D is now shifted a short distance along the wire and the whole procedure is repeated, the magnitude of

the movement of the mercury being particularly noted. From a comparison of the results of these two observations it will be obvious in which direction the slider must be moved in order to obtain a smaller deflection. The slider is moved in this direction and a further observation is made, and so on, until the direction of the movement of the mercury is reversed, the object being to find the point at which the deflection is zero. As soon as the deflection of the electrometer has been reduced to 3–4 scale divisions of the eye-piece micrometer the side circuit can be kept closed for a rather longer time in order to facilitate the observation, and when the deflection has become only just detectable, but only then, the following procedure is best adopted for determining the exact zero point:—1. Open the short-circuiting key of the electrometer. 2. Close the side circuit for 2–3 seconds, and, while viewing the meniscus through the microscope, suddenly close the short-circuiting key again. The return movement of the mercury towards its equilibrium position will be observed, and as this takes place more rapidly than the original deflection it is more easily detected. The side circuit is broken again as soon as possible. By working in this way it is possible to define the position of the point of balance within a fraction of a millimeter.

A really sensitive electrometer should show a deflection of from 2 to 4 scale divisions when the slider is 1 mm. from the point of balance. We thus get an opportunity of testing the sensitiveness of the instrument.

Calculation of the result. Since the combined resistances of the leads AB and AC are negligible compared with the resistance of the potentiometer wire there exists the same difference of potential between the points B and C as between the terminals of the accumulator (E_{acc}). When the point of balance D has been obtained the potential difference between D and C (E_{DC}) is equal to the E.M.F. of the cadmium cell (E_{cad}).

Now: $E_{DC} : E_{BC} =$ Length DC : Length BC.

The desired E.M.F. of the accumulator E_{BC} is therefore given by

$$E_{BC} = E_{DC} \cdot \frac{\text{Length BC}}{\text{Length DC}}$$

or

$$E_{acc} = E_{cad} \cdot \frac{BC}{DC}.$$

(It should be noticed that it is the ratio BC : DC that is important in this connection; in the case of the measurement of the electrical conductivity it is the ratio BD : DC whose value is required.)

For example, if

$E_{cad} = 1{\cdot}0185$ volts., BC = 1000 mm. and DC = 555·5 mm.

then

$$E_{acc} = \frac{1{\cdot}0185 \times 1000}{555{\cdot}5} = 1{\cdot}8335 \text{ volts.}$$

EXERCISE 69.

Measurement of Electromotive Forces by means of Resistance Boxes and a Regulating Resistance.[1]

Reading directly in Millivolts.

In cases where a large number of measurements is to be carried out the following procedure is to be recommended: For the potentiometer wire BDC shown in fig. 27 two resistance boxes arranged as indicated in fig. 28 are substituted. Each box has resistances of 1, 2, 2, 5, 10, 20, 20, 50, 100, 200, 200, 500 ohms respectively, making a total of 1110 ohms. To commence the determination all the plugs are removed from the box on the left. If now the 50 ohm plug, for example, of the box on the right is transferred to the corresponding position in the left-hand box the effect is the same as if a sliding contact on a potentiometer wire 1110 mm. in length had been moved 50 mm. along from its extreme right-hand end. Every ohm that is transferred from the right to the left box leads to an increase of the potential difference between the points D and C by $^1/_{1110}$ of the whole E.M.F. of the accumulator. The potential difference between D and C is therefore proportional to the total value of the resistance plugs that have been transferred from the right to the left box. It is possible for the sake of convenience so to adjust the value of the proportionality factor that each ohm corresponds to a change of potential of 1 millivolt. For this purpose it is necessary merely to reduce the effective E.M.F. of the accumulator by means of an adjustable resistance inserted in the circuit between A and B until a point of balance against the cadmium cell is obtained when plugs corresponding to 1018·5 ohms

[1] L. Michaelis, *Die Wasserstoffionenkonzentration*, 2nd edn. Berlin, Julius Springer (1922).

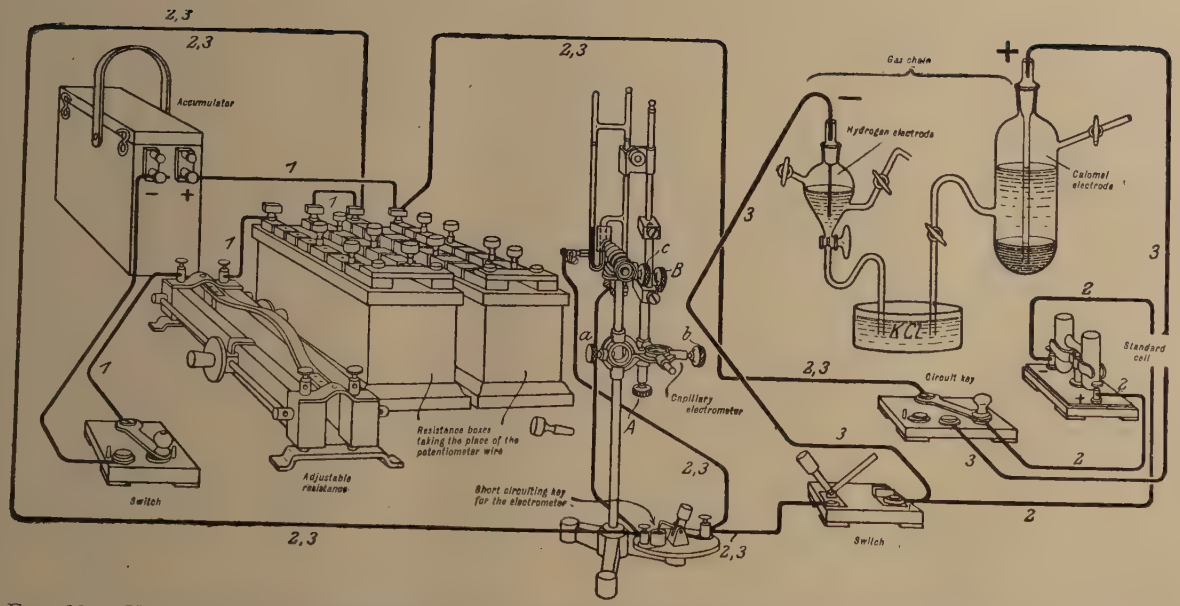

FIG. 28. — Half-schematic representation of the Compensation Circuit with resistance boxes and adjustable resistance. The E.M.F. under measurement is furnished by a "Gas-chain" composed of a Hydrogen Electrode (pear-shaped form) and a Calomel Electrode.

have been transferred from the right to the left box. The adjustable resistance (fig. 28) should have a maximum value of 1500–2500 ohms, and should possess both coarse and fine adjustments, but it need not be calibrated in any way. The procedure is then as follows:

All the plugs are removed from the left resistance box and then plugs corresponding to an aggregate value of 1018 ohms are transferred from the right box to corresponding positions in the left. The adjustable contact of the regulating resistance is placed at about its mid-point and a reading is taken, as previously described, with the capillary electrometer. The setting of the regulating resistance is then varied until the point is found at which there is an exact balance. A more exact adjustment is made by finding the point at which a small deflection is obtained in one direction when 1018 ohms have been transferred, and a similar deflection, but in the opposite direction, when 1019 ohms have been taken across. By working in this way it is unnecessary to know the E.M.F. of the accumulator; the variable resistance is kept adjusted so that the above condition is fulfilled. Then each ohm that must be transferred in order to produce a point of balance when, in the experiments to be described, an unknown E.M.F. is substituted for the cadmium cell corresponds to exactly 1 millivolt of the E.M.F. that is being measured. Tenths of a millivolt may be estimated. This completes the apparatus required for the measurement of any desired electromotive force.

EXERCISE 70.

Preparation of Calomel Electrodes and Chlorine Ion Concentration Cells.

A calomel electrode consists essentially of a mass of mercury in contact with a fluid saturated with calomel. The potential of such an electrode depends on the exact composition of this fluid. We will use first a ·1 N. solution of KCl. The purest KCl (Kahlbaum's " for analysis ") is heated to dull redness for a short time. After cooling in the desiccator 74·56 gms. of it are dissolved in water and made up to a 1 litre in a measuring flask. This gives a normal solution from which the ·1 N. solution is made by careful dilution of a portion to exactly 10 times its volume.

The electrode vessel is shown in the accompanying figure 29.

Pure mercury (for the method of purification see p. 150) is poured in until the platinum contact wire is well covered. It is advisable previously to amalgamate this wire, otherwise it is easy for a capillary layer of the upper aqueous fluid to creep in between the platinum and the mercury, and this might lead to an error of several millivolts in the potential of the electrode or to a lack of stability of the potential. The process of amalgamation is carried out as follows: The platinum wire is removed, cleaned in strong sulphuric acid, well washed, and then made the cathode in a 1 per cent. solution of mercurous nitrate acidified with a few drops of nitric acid and contained in a separate vessel; a small platinum electrode will serve as anode. The current from a single cell (2 volt) accumulator is then passed through just long enough to coat the platinum wire with a grey layer of mercury—about 1 minute. If the current is too strong, or if it is passed for too long a time the mercury will not adhere to the platinum, but will fall off in the form of bright metallic drops. The amalgamated platinum is now washed in water, dried by means of filter paper, and then replaced in the electrode vessel, the stopper being lightly greased.

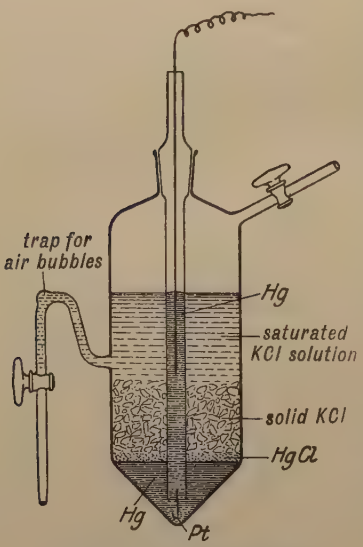

FIG. 29.—Calomel Electrode.
The filling is that of the saturated calomel electrode (see exercise 75). In the present case ·1 N. KCl is to be used instead of the saturated KCl solution mentioned in the figure, and the layer of solid KCl is entirely omitted.

A small spoonful of calomel (the medicinal preparation or that supplied by Kahlbaum) is now placed in a dish and washed with about 30 c.c. of the KCl solution that is to be used later for filling the electrode (in this case the ·1 N. solution). After being stirred for several minutes with a glass rod the calomel is allowed to settle and the upper liquid is poured off as completely as possible. This washing is repeated 5 to 6 times. The calomel is then transferred with some of the KCl solution to the electrode vessel, best by

suction through the side tube so as not to disturb and possibly soil the platinum contact. When this calomel has settled (only the thinnest layer is needed) the vessel is sucked full of the ·1 N. KCl solution, care being taken that the side contact tube is free from air-bubbles. The tap is then closed and the electrode is supported in a stand with its contact tube below the surface of a further portion of the ·1 N. KCl solution.

We will now make a concentration cell with this calomel electrode and a second made up with a different solution. The solution to be used for the second is as follows: 1 N. KCl solution 2·00 c.c., 1 N. KNO_3 solution (made up with Kahlbaum's salt " for analysis," completely free from Cl), 18 c.c., and distilled water to bring up the volume to 200 c.c. We shall describe later the significance of the use of this particular mixture.

This second electrode is filled in a similar way to that used for the previous one, the calomel being washed with some of the new solution. But it is also permissible, in order to economise in the use of a costly standard solution to carry out the washing of the calomel 5 or 6 times with distilled water and then to follow this by three washings only with the particular fluid to be used in the electrode. Further, the dimensions of the electrode vessel may be reduced so that while retaining the same form its capacity is only about 1–1·5 c.c., then from 5 to 10 c.c. of solution will suffice for both washing the calomel and filling the vessel.

Both these calomel cells are mounted in a stand so that their side openings are immersed in the same vessel of saturated KCl solution. The " concentration cell " so formed is then inserted in the arrangement for the measurement of E.M.F. in place of the cadmium cell, and in such a way that the electrode that contains the smaller concentration of Cl corresponds to the positive pole of the cell. The E.M.F. of the arrangement is then measured.

If the slide wire (fig. 27) is used the sliding contact is shifted along until the point of zero deflection is obtained. Then the E.M.F. of the concentration cell is given by

$$E = E_{acc} \cdot \frac{DC}{BC}$$

If resistance boxes and a regulating resistance are used the E.M.F. is simply numerically equal to the number of ohms that have been plugged over from the right to the left box.

The result should be ·0577 volts. if the experiment is carried out at 18° C., or, in general,

at 15° C. ·0571	at 20° C. ·0581
,, 16° C. ·0573	,, 21° C. ·0583
,, 17° C. ·0575	,, 22° C. ·0585
,, 18° C. ·0577	,, 23° C. ·0587
,, 19° C. ·0579	

The maximum permissible limits of error are $\pm$ ·0005 volt., but a beginner may be content with values within $\pm$ ·001 volt. For example, a value of ·0575 volt. at 16° C. would be satisfactory.

The potential should be measured every 5 minutes until three consecutive readings give the same value. This constant value must also be maintained for two hours subsequently. If the experiment has been carried out properly, and particularly if the platinum contact below the mercury has been amalgamated and has not been uncovered during the filling of the electrodes, the definitive value of the potential should be established with great precision right from the beginning of the observation; but sometimes the attainment of the final value is slower and will require as much as an hour. But in any case the readings must approach continuously nearer to the final value provided that the electrodes are kept completely at rest; if it is not so then errors due to uncertain contacts or impurities in the mercury or some such cause are creeping in and the experiment must be rejected.

The above solutions were used for this exercise because the result obtained with them is of theoretical importance. The relation of the chlorine contents of the two solutions is 1 : 10. Since they both possess the same concentration of potassium ions the degree of dissociation of the KCl is the same in both cases so that the ratio of their Cl′-ion concentrations is also 1 : 10. According to Nernst the E.M.F. of a concentration cell is given by

$$E = RT \log_e \frac{c_1}{c_2},$$

where c_1 and c_2 are the concentrations of the ions responsible for the production of the E.M.F., R is the gas constant and T is the absolute temperature. Using common logarithms and calculating for a temperature of 18° C. this formula reduces to

$$E = \cdot 0577 \cdot \log_{10} \cdot \frac{c_1}{c_2} \text{ volts.}$$

In the particular case where $c_1 : c_2 = 10 : 1$, $\log_{10} \frac{c_1}{c_2} = 1$ and E = ·0577 volts. It is the object of the above exercise to introduce this important constant.

The ions that are actually responsible for the establishment of the E.M.F. are not the Cl′ ions, but the Hg ions from the dissolved calomel. But as the solubility of the calomel is inversely proportional to the Cl′ ion concentration the observed E.M.F. may be referred with equal exactness to the relative concentrations of the Cl′ ions. This is an example of an electrode of the second order.

As a second example of Cl′ ion concentration cell we will combine a ·1 N. calomel electrode with one filled with the following mixture:

·1 N.KCl solution	·25 c.c.
1 N.KNO_3 solution	20 c.c.
Cl-free distilled water	to bring up the volume to 200 c.c.

This fluid is a ·000125 N. solution of KCl in ·1 N. KNO_3. Since its potassium content is practically the same as that of the ·1 N. KCl solution the degree of dissociation of the KCl in each fluid will also be the same. The Cl′-ion concentrations in the two electrodes will therefore be proportional to their KCl concentrations. Let us first consider the value that we should expect the E.M.F. of such a cell to possess at a temperature of, for example, 17·5° C. As before, the equation is

$$E = \cdot 0576 \cdot \log_{10} \frac{c_1}{c_2},$$

where c_1 and c_2 are the respective concentrations of KCl. In this case therefore $\frac{c_1}{c_2} = \cdot 00125$, so that $E = \cdot 0576 \times (\cdot 097 - 3) = - \cdot 1672$ volt. The following are actual experimental values, the solution of smaller Cl′-ion concentration being taken as the positive pole:

Immediately after setting up the cell	·1635 volt.
After 15 minutes	·1655 ,,
,, 30 ,,	·1660 ,,
,, 1 hour and subsequently constant	·1666 ,,

Thus the value agrees within half a millivolt with the calculated E.M.F. The negative sign before the calculated value arises from the arbitrary definition of the polarity. If we regard c_1 as the

greater and c_2 the lesser concentration of Cl′-ions we obtain $+\cdot1672$ volt, since

$$\log. \frac{c_1}{c_2} = -\log. \frac{c_2}{c_1}.$$

The principle of this exercise can also be applied in the inverse manner, and these concentration cells can be used for the determination of Cl′-ion concentrations.

EXERCISE 71.

Electrometric Determination of Chlorine Ion Concentrations.

As unknown solution it is convenient to use a solution made up by another worker by diluting the ·1 N. KCl solution, so that the final strength is somewhere between ·1 and ·0001 N. Naturally a fairly large quantity of this dilute solution will be made up, but one should restrict oneself to about 8 or 10 c.c. for the analysis in order to satisfy the conditions of a micro-analysis.

The unknown solution is first diluted with $^1/_9$ of its volume of 1 N. KNO_3 solution that has been tested and found free from chlorides; the object of this is to bring the degree of dissociation (or, in terms of Bjerrum's theory of ionic activities, the " activity of the chlorine ions ") to the same value as in the ·1 N. KCl solution with which the second comparison electrode is to be filled. Some calomel is now washed some 8 to 10 times with distilled water. It will suffice if after this washing a small knife point of the solid remains. This is now washed 3 to 4 times with successive 1 or 1·5 c.c. portions of the solution under investigation, care being taken to ensure the efficiency of this process by vigorous stirring and as complete sucking off of the fluid as possible. Finally, the remainder of the fluid is poured on to the calomel and the mixture is sucked into a micro-electrode, which has already been provided with a layer of pure mercury. The vessel should have the form shown in fig. 27, but without the tap on the side contact tube; its capacity should be about 1 to 1·5 c.c. When the calomel in the electrode has settled the upper clear fluid is again blown out into the same dish and once more drawn into the electrode with a further quantity of calomel, and this is repeated until after settling a uniform if thin layer of calomel covers the mercury. It is, as a matter of fact, inadvisable to have a thick layer of calomel on the surface of the mercury as otherwise there is a greater danger of soiling the

platinum contact wire. The micro-electrode is now combined with a ·1 N. electrode in the way described in Exercise 70 in order to form a cell whose E.M.F. is to be measured.

It is recommended not to immerse the contact tube of the micro-electrode directly in the saturated KCl solution as if a diffusion current carried a little of this strong solution into the small volume of fluid in the electrode a considerable change of Cl-ion concentration would result and the determination would be spoiled. In this case it is better to use a saturated solution of ammonium nitrate, or if some of the mixture of the original solution with KNO_3 is still left the micro-electrode can be immersed in this. The tube of the ·1 N. electrode is then to be immersed in a vessel of ·1 N. KCl and the cell completed by means of a glass syphon tube containing a saturated solution of KCl in 3 per cent. agar made by heating the ingredients together in a double saucepan.

The E.M.F. is measured as described in Exercise 70. The calculation is made by means of the formula:

$$E = \cdot 0577 \log. \frac{c_1}{c_2}$$

if the measurement is carried out at 18° C. In other cases the appropriate value of the constant is obtained from the table on page 162 c_1 is the concentration of the known and c_2 that of the unknown solution. We can write the formula in the form

$$\log. \frac{c_1}{c_2} = \frac{E}{\cdot 0577}.$$

Suppose, for example, that a value for E of ·1646 volt. was observed at 16·5° C. Then

$$\log. \frac{c_1}{c_2} = \frac{\cdot 1646}{\cdot 0574} = 2 \cdot 868$$

so that

$$\frac{c_1}{c_2} = 738$$

c_1 is ·1 N. so that $c_2 = \frac{1}{7380}$ N. = ·0001356 N.

This result must now be increased by 10 per cent. of its value in order to allow for the preliminary dilution with the KNO_3 solution. The final result then comes to be ·000149 N. As a matter of fact in the experiment from which these data were obtained the unknown fluid was a ·000138 N. solution of KCl so that the experimental error amounts to + 8 per cent.

An error of 2 millivolts in the measurement of the E.M.F. will lead to an error of about 10 per cent. of the total value, and such an error may easily occur in either direction in this micro-method, so that an error of $\pm$ 10 per cent. must be regarded as unavoidable, and an inexperienced worker will often obtain errors of double this magnitude. The Cl′ ion concentration here chosen is not far removed from the lower limit of concentration that can be estimated with any degree of accuracy. If only 5 c.c. of the solution are used, and the measurement can be carried through easily with this quantity, ·000,002 gm. of Cl may be taken as the smallest quantity that can be estimated with the degree of accuracy mentioned.

If a new measurement is to be made with the same electrode it is absolutely necessary to take the whole to pieces and to clean and dry it before re-filling. Special care should be taken with the platinum contact wire; it is strongly recommended that this should be re-amalgamated as described on page 160 for each experiment.

EXERCISE 72.

Measurement and Experimental Elimination of Diffusion Potential.

At the junction between two liquids of different compositions a difference of potential is set up whose value depends on the quantity and nature of the electrolytes present. Since this potential can only be measured by leading off with metallic electrodes, which themselves will establish their own potentials with respect to the different fluids, the diffusion potential as a rule will constitute only one member of the series of separate potentials that make up the total potential of the cell. The present exercise, however, furnishes a case in which the diffusion potential can be measured alone.

A first calomel electrode is filled with ·1 N. KCl and a second with ·1 N. HCl. Since the concentrations of Cl′ ions are the same in both electrodes they will cancel out, leaving only the diffusion potential effective. The side contact tubes of the electrodes are immersed in a dish of one of the fluids—it is immaterial which—and the potential difference between them is measured. At 18° C. the value to be expected is given by

$$E = \cdot 0577 \cdot \log. \frac{u_1 + v_2}{u_2 + v_1} \text{ volts.}$$

where u_1 and v_1 are the mobilities of the cation and anion respectively

of the one electrolyte and u_2 and v_2 the corresponding values of the ions of the other. The values of these quantities in the case of the electrolytes we are using in this experiment are as follows:

for HCl $u_1 = 330$ $v_1 = 65$
for KCl $u_2 = 65$ $v_2 = 65$,

so that $E = \cdot0577 \cdot \log. \frac{395}{130} = \cdot0271$ volt.

This value will be readily confirmed by experiment to within 1 millivolt. (For example, the actual E.M.F. observed may be ·0275 volt.)

If now the simple modification is introduced of using as connecting fluid between the two electrodes not the solution present in one of them, but instead a saturated solution of KCl, it will be found that the E.M.F. of the combination has now sunk to ·0015 volt. The interposition of saturated KCl solution diminishes all diffusion potentials. The same is not true of a solution of NaCl because, while the mobilities of the ions K and Cl are about equal, those of the ions Na and Cl are appreciably different.

A diffusion potential of 27 millivolts such as we have observed in this exercise is greater than those that are ordinarily met with. And in general a diffusion potential is practically completely eliminated by a saturated solution of KCl.

EXERCISE 73.

The Membrane Potential of the Skin of an Apple.[1]

An undamaged apple is selected, and from one side a small cap of the skin is removed. The uncut side is then immersed in a shallow layer of ·001 N. KCl solution. One calomel electrode is then immersed in this solution at an appropriate distance from the apple and a similar electrode is brought into contact with the upper cut surface of the fruit. A potential difference of about ·09 volt. will be observed, the cut surface of the apple being the negative pole.

If now the experiment is repeated with the intact portion of the apple skin immersed in ·01 N. KCl the voltage observed will be about ·05 volts.; while if ·1 N. KCl solution is used the reading will be in the neighbourhood of ·01 volt.

[1] Jacques Loeb and R. Beutner, *Biochem. Zeitschr.* **41,** 1. 1912. For the complete interpretation of this fundamental experiment it is particularly recommended to consult R. Beutner's *Die Entstehung elektrischer Ströme in lebenden Geweben,* Stuttgart, 1920.

Thus it is seen that at the junction between the apple skin and KCl solution there is set up a potential difference whose value depends on the concentration of the solution. If in two experiments the ratio of the KCl concentrations is 1 : 10 the potentials observed in these two experiments differ by the same amount as do those of two calomel electrodes whose KCl concentrations are in the same ratio—i.e. by ·0577 volt.

The potential difference between the exposed tissues of the apple and the outer surface of its skin has no definite fixed value, but is influenced by the composition of the solution in contact with the membrane. The same is also true in the case of animal membranes, but when these are used the change of potential observed for a 10-fold change of concentration of the outer fluid is much less than the expected amount of ·0577 volt.

EXERCISE 74.

Hydrogen-ion Concentration Cells with streaming Hydrogen.

Platinum black that is saturated with hydrogen gas possesses the same electromotive properties as would be expected of an electrode composed of metallic hydrogen. By taking advantage of this fact hydrogen ion concentration cells can be constructed. The exact form of electrode used will vary according to the particular circumstances of each experiment. As a first exercise we will measure the E.M.F. of a H·-concentration cell containing the following solutions.

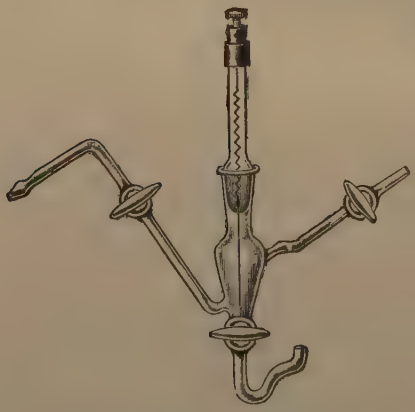

FIG. 30.—Hydrogen Electrode, pear-shaped form. $^1/_3$ natural size.

1. ·5 c.c. N. HCl + 19·5 c.c. N. KCl + 180 c.c. of distilled water, i.e. a mixture that is ·0025 N. with respect to HCl and has a total Cl′ concentration of ·1 N.

2. 50 c.c. of 1 N. NaOH + 100 c.c. 1 N. acetic acid + 350 c.c. of distilled water, i.e. a solution that is ·1 N. with respect to both acetic acid and sodium acetate (standard acetate).[1]

[1] L. Michaelis and A. Gyemant, *Biochem. Zeitschr.* **109**, 165. 1920.

Pear-shaped electrode vessels of the form illustrated in fig. 30 are used. The platinum electrode itself is in the form of a wire and is fused into the ground-in glass stopper. Before use it must be coated with platinum black ·1 gm. of platinum chloride + ·007

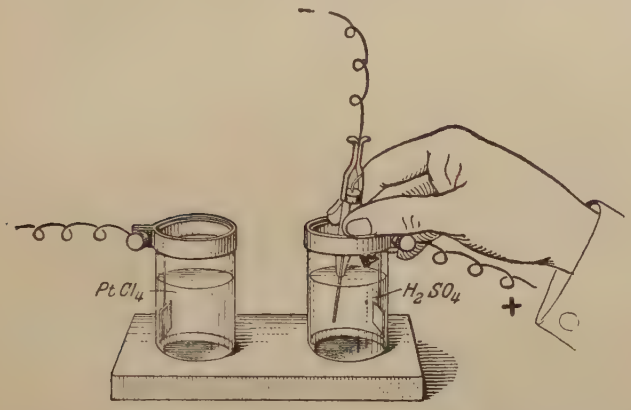

FIG. 31.—Platinising and Reduction of a Platinum Electrode.

gm. of lead acetate are dissolved in 30 c.c. of water and 10 c.c. of this solution are placed in a small vessel such as is shown in fig. 31, or where the greatest economy of platinum must be observed of the equally suitable form illustrated in fig. 32. A small auxiliary electrode of platinum is connected to the positive pole of a four-volt accumulator, and is then immersed in the liquid. The electrode to be platinised is first cleaned with strong sulphuric acid, well washed with water, and then connected with the negative pole of the accumulator and also immersed in the solution. A moderate evolution of gas takes place and the electrode becomes covered with a velvety black layer of platinum black. A new electrode will require as much as 5 minutes' treatment, but one that has been platinised previously will be ready in one minute. The blackened electrode is now well washed with water and then used as cathode in dilute sulphuric acid, the connections being the same as those used during the platinising. A vigorous evolution of gas will now occur. Care

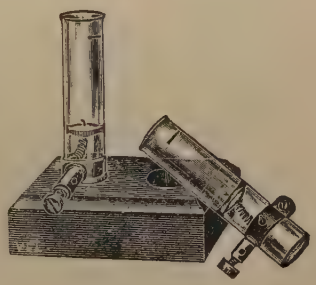

FIG. 32.

must be taken to pass the current in the appropriate direction (see fig. 31).

The electrode is now removed from the acid and again well washed in water; its stopper is dried and lightly greased and then inserted air-tight into the electrode vessel. Finally the vessel is rinsed with several changes of water during the course of an hour, after which it is ready for use.

As a general rule the electrode will require to be replatinised every few weeks; it should be kept immersed in distilled water when not in use.

One such electrode is loaded with one of the solutions under investigation, and a second electrode with the other solution, by suitably adjusting the taps and then sucking up the liquid from a small dish. The first portion sucked in is run out again immediately and this washing out of the vessel is repeated twice more before the final portion of the solution is taken in. Care must be taken to ensure that the outflow tube is quite free from air-bubbles, especially just below the tap. The amount of fluid finally retained in the electrode should be such that the platinum electrode is half immersed. The vessel can now be mounted in its stand with the outflow tap kept closed for the time being. The hydrogen is now introduced. This must be prepared[1] from arsenic-free zinc and dilute sulphuric acid with the addition of a little $CuSO_4$ or a drop of platinum chloride, and washed successively with 2 per cent. $KMnO_4$, then with a strong solution of mercuric chloride, and finally with caustic soda. This latter absorbs the traces of CO_2 formed in the permanganate flask by the oxidation of organic vapours; this is particularly necessary when strongly alkaline solutions are being investigated. The hydrogen passes in through the tap on the left side (fig. 30), and is led off from the tap on the right. As it bubbles through the fluid it carries off any oxygen that may be in solution, itself dissolves to saturation and fills the upper gas space. The stream of gas should be fairly brisk—3 to 5 bubbles a second. After about 3 minutes first the exit and then the inlet tap is closed, and the hydrogen generating apparatus is

[1] Pure hydrogen is very conveniently prepared by the electrolysis of 10 per cent. caustic soda solution between electrodes of sheet nickel. The vessel used must be large enough to carry a current of 3–4 amps. without undue heating. Traces of oxygen are removed by passing the gas through a warmed tube containing palladiumised asbestos. For further details see Clark's *The Determination of Hydrogen -ions* referred to on p. 39.—*Translator.*

disconnected and the electrode is inverted some 30–50 times in its rotating stand (fig. 33) in order to accelerate the attainment of equilibrium between the solution and the hydrogen atmosphere.

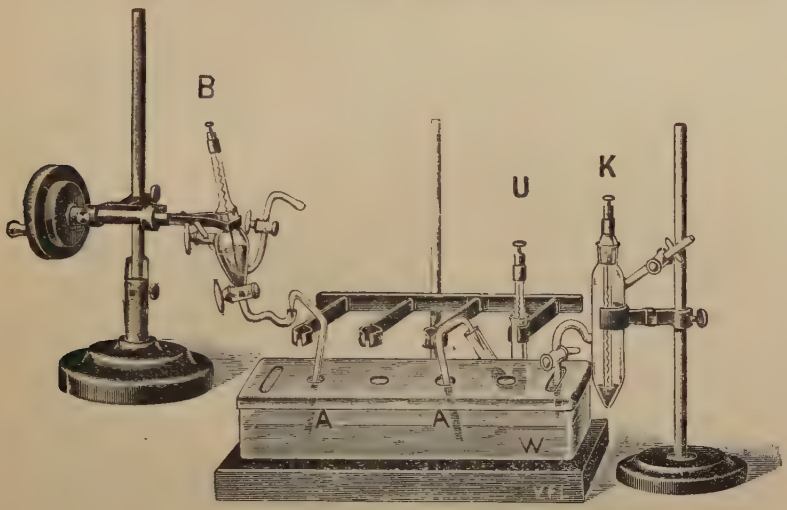

FIG. 33.—Electrodes and Stand.

B, pear-shaped electrode and rotating stand. K, calomel electrode. U, U-shaped electrode. A, KCl-agar tube for connecting the gas electrodes to the bath of saturated KCl.

It might have been thought that the bubbling of the gas through the liquid would have sufficed for this, and as a general rule this will be the case. But the rotation does markedly increase the exactness and the rapidity of the establishment of the potential. The reason undoubtedly is that if the hydrogen is contaminated with the smallest trace of an electrolytically active gas such as oxygen while a prolonged bubbling through the solution will be of no use, the shaking of the enclosed gas will give an opportunity for the catalytic reduction of this oxygen on the platinum surface to water, so that the disturbing factor is removed.

The other electrode containing the second solution is treated in exactly the same way.

After a few minutes the passage of the hydrogen is repeated, and finally a third portion is allowed to pass through. The lower tap is now opened and the open end of the outflow tube is connected to a dish of saturated KCl solution by means of a tube of saturated

KCl-agar. The form of this tube is shown in fig. 33, A, and its mode of preparation is as follows:

A small sharply bent glass tube drawn out at one end to a short, fine point is filled free from air-bubbles with a still warm solution of 3 gms. of agar + 40 gms. of KCl in 100 c.c. of water prepared by boiling these constituents for a considerable time on the water bath. Having been filled the tube should be preserved in saturated KCl solution until required for use when it is dried and so arranged that its tip dips into the electrode fluid while its lower end dips into a vessel of saturated KCl solution. Into the same vessel the similar agar connecting tube from the second electrode also dips. It is recommended to drop a little finely powdered solid KCl into the upwardly directed ends of the electrode tubes; this renders more complete the elimination of the diffusion potential and ensures the maintenance of the saturation of the agar tips.

The concentration cell so made up is connected up to the compensation circuit for the measurement of E.M.F. in place of the cadmium cell and in such a way that the HCl solution corresponds to the positive pole and the standard acetate to the negative. The E.M.F. is to be measured every 5 minutes until a constant value is obtained. This definitive value must be almost if not quite completely attained at the beginning; if this condition does not obtain the saturation with hydrogen is not complete and must be repeated. At 18° C. the E.M.F. of this cell is ·1140 volt., and with careful working the maximum permissible limits of error are ±·7 millivolt.

NOTE.—If two pear-shaped electrodes are not available the procedure described in the next exercise can be employed; the electrode is filled with the first solution and the potential is measured against the saturated calomel electrode; the same pear-shaped electrode is now filled with the second solution and the potential against the saturated calomel is again measured; the difference between these results is the value that would have been obtained if two electrodes filled respectively with the two solutions had been measured against each other.

Now, since we know the h of the HCl solution we have employed, we can calculate that of the standard acetate solution.

At 18° C. $$E = \cdot 0577 \log. \frac{h_{HCl}}{h_{acet.}}$$

or $$\log. \frac{h_{HCl}}{h_{acet.}} = \frac{\cdot 1140}{\cdot 0577} = 1 \cdot 976.$$

or $$\frac{h_{HCl}}{h_{acet.}} = 94 \cdot 6.$$

If the HCl were completely dissociated $h_{HCl} = \cdot0025$. We now assume that the degree of dissociation in a weak solution of HCl containing an excess of KCl is the same as in a pure solution of HCl of the same Cl-content, i.e. in a ·1 N. solution of HCl. This degree of dissociation is determined by comparing the molecular conductivity of HCl in ·1 N. solution with that at infinite dilution, and has been found to possess a value of ·917. Therefore in our solution $h_{HCl} = \cdot0025 \times \cdot917 = \cdot00229$ N., so that

$$h_{acet.} = \frac{\cdot00229}{94\cdot6} = 2\cdot42 \times 10^{-5} \text{ and } pH = 4\cdot616.$$

NOTE.—As we mentioned before, it has become very doubtful whether it is permissible to deduce the value of h for a dilute solution of HCl directly from its conductivity. Bjerrum estimates the H·-ion activity, a_h, ofthe HCl solution we have employed at ·00202 instead of ·00229. In this case the a_h of the standard acetate would be $\frac{\cdot00202}{94\cdot6} = 2\cdot14 \times 10^{-5}$ and $pH = 4\cdot67$ instead of 4·616. From this it would seem that all the results of pH measurements that have been made hitherto should be corrected by the addition of ·05. Such a correction has not, however, been introduced in this book; it becomes of special importance in the calculation of the ionisation constant of water.

EXERCISE 75.

Preparation and Calibration of a Saturated Calomel Electrode.[1]

In order to measure the hydrogen ion concentration of an unknown solution some form of standard electrode is required. In the preceding exercise we used a hydrogen electrode in a solution of HCl for which h = ·00229 N. For simplicity of calculation an acid solution in which h = 1 would have been preferable, but this would not be so suitable in practice on account of the large diffusion potential that would be set up. But it is not by any means essential that the standard electrode should be a hydrogen electrode; any metallic electrode will serve provided that it is permanent and does not require to be refilled each time before use. The most convenient for the purpose is the saturated calomel electrode which is prepared

[1] L. Michaelis, *Die Wasserstoffionen-Konzentration*, Berlin, Julius Springer, 1st. Edn. 1914.

in a way similar to that employed for the ·1 N. electrode (p. 159), using as liquid a saturated solution of KCl and adding a thick layer of solid KCl above the calomel. The side tube of such an electrode is best provided with a tap that can be kept permanently closed, the saturated KCl solution in the capillary space between the barrel and the stopper of the tap being found to be sufficient for the conduction of the current. The tap serves simply to provide an exit for the solution when it is necessary to expel an air bubble from the side tube. The tip of the exit tube should be kept continuously below the surface of saturated KCl solution. With these precautions the electrode will last for years. Against the normal hydrogen electrode it shows a potential difference of ·2500 volt. at 18° C.; but as different electrodes show small variations between themselves, and even the same electrode will vary slightly from time to time, no great reliance is placed on this value, but the electrode is calibrated occasionally by measuring its potential against a hydrogen electrode containing standard acetate. Suppose that at 18° C. this potential is found to be ·5160 volt., we must now calculate the value of the potential difference that would be observed between the electrode and a hydrogen electrode in which the solution had a concentration of 1 N. in respect of hydrogen ions. In Exercise 74 we found the pH of the standard acetate solution to be 4·616. The potential difference between the standard acetate and the 1 N. hydrogen-ion solution at 18° C. is therefore

$$E = 4{\cdot}616 \times {\cdot}0577 \text{ volt.} = {\cdot}2663 \text{ volt.}$$

Consequently the potential difference between the calomel electrode and the normal hydrogen electrode is ·5160 − ·2663 = ·2497 volt.

For practice in the use of this electrode the potential difference between a saturated calomel electrode that has been calibrated in this way and a hydrogen electrode filled with a mixture of equal parts of M/15 primary potassium phosphate and M/15 secondary sodium phosphate (see Exercise 13, p. 33) may be measured. Suppose the value obtained is E = ·6426 volt. Then the potential difference between the phosphate mixture and the normal hydrogen electrode is

$$\cdot6426 - \cdot2497 = \cdot3929 \text{ volt., so that the } \mathrm{pH} = \frac{\cdot3929}{\cdot0577} = 6{\cdot}81.$$

From what we have already said we may summarise the following

general directions for the most convenient procedure for the measurement of the pH of any given liquid.

1. First, the potential between the saturated calomel electrode and a hydrogen electrode containing standard acetate must be measured for the day of the experiment. Let this value be denoted by E_o.

2. Then measure the potential difference given by the same calomel electrode and a hydrogen electrode containing the solution under investigation. Let this be E_x.

3. Calculate the value of the difference $E_o - E_x$ in millivolts and divide the result by D. The value of D depends on the temperature, and is to be obtained from the table on p. 162. The number obtained in this way must be added algebraically to 4·62 (i.e. subtracted from 4·62 should its value be negative). The final result is the pH of the solution under investigation.

If a number of pH measurements are to be made on the same day the value of E_o for that day can be determined once for all and used as the basis for the calculation of all the results. E_o is found to vary from 514 to 517 millivolts according to the temperature and the uncontrollable variation of the calomel electrode. The procedure recommended here is based on the observation that the hydrogen-standard acetate electrode is more exactly reproducible than the calomel electrode and on the consideration that all corrections for temperature and barometric pressure are eliminated. It combines the technical advantages of the immediate availability of the calomel electrode with the theoretical superiority of a hydrogen electrode.

EXERCISE 76.

Hydrogen Electrode with Stationary Gas Bubble[1]. Measurement of the pH of serum.

In the case of fluids that contain CO_2 and whose hydrogen ion concentration would be diminished by the passage of a stream of hydrogen, another form of electrode vessel is required. The U-shaped electrode about to be described will serve in such cases,

[1] L. Michaelis, l.c.

and indeed can be used for pH measurements generally, but it requires a longer time for the establishment of the definitive potential (fig. 34).

The platinising is carried out as already described. The vessel is filled as follows: The fluid is introduced until the shorter limb is completely and the longer limb partly filled. The hydrogen—prepared and purified as above described—is allowed to stream through a fine capillary glass tube 5 cms. long for a sufficient length of time to ensure that all the air is displaced. The end of the capillary is now brought up to the electrode and plunged below the surface of the liquid. At the same instant the connecting rubber tube is compressed by means of the finger just above the capillary in order to cut off the stream of gas and so to prevent the washing out of the CO_2 and the consequent change of pH. The capillary is now pushed down to the lowest part of the bend, and by gently raising the finger small bubbles of hydrogen are allowed to ascend to the platinum electrode until only its extreme tip is immersed in the fluid. If foaming takes place this is of no consequence. The capillary is then withdrawn, the longer arm of the U-tube is filled completely with the solution and the stopper is inserted without permitting air bubbles to enter. When so loaded the vessel is tilted backwards and forwards either by hand or by means of the rotating stand, fig. 33, until the hydrogen bubble has passed 50 times along the whole length of the U-tube and back again. In order to maintain the pressure inside at atmospheric the glass stopper is given an occasional complete turn at a time when the hydrogen bubble is at the platinum end of the vessel; this places the appropriate bore of the stopper into momentary communication with the outside air. When the electrode has been reversed the required number of times the bubble is brought finally to the platinum electrode; the stopper is then removed and the apparatus is mounted on a stand. The fluid junction is established by means of an agar-KCl tube prepared according to the directions on page 172.

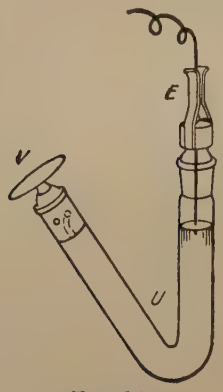

FIG. 34.
U-shaped Electrode.

Blood serum is used in this experiment as the unknown solution. The comparison electrode is a saturated calomel electrode which

forms the positive pole of the combination. The values obtained for the pH of the serum will vary according to the amount of CO_2 that the fluid has lost while standing; the usual limits are between 7·6 and 7·7, which correspond to a potential against the saturated calomel cell of about ·69 volt.

EXERCISE 77.

Measurement of the Hydrogen-Ion Concentration of Blood.[1]

The following method is the easiest for the measurement of the reaction of blood, and depends on the fact that blood suffers no appreciable change of pH when it is diluted with CO_2-free NaCl solution. ·85 per cent. NaCl solution is boiled in a Jena flask, and is then cooled out of contact with the air. This solution is then placed in a U-shaped electrode in such quantity that the longer limb is but half filled. A bubble of hydrogen is added in the usual way, but is not shaken with the liquid. A few grains of hirudin or of sodium oxalate having been placed in the empty part of the longer limb the blood is drawn from the elbow vein of the subject into a syringe, and is at once transferred to the electrode until the latter is completely filled. The stopper is then inserted and the shaking proceeded with in the usual way. Should a clot form on the platinum electrode no constant potential, or only an apparently constant false potential, will be established.

Otherwise the definitive potential is rapidly set up and then remains constant. The reading should be repeated at five minute intervals after the setting up of the gas chain until 3 successive readings agree.

For human venous blood at 18° C. an E.M.F. of ·674 volt. will ordinarily be observed, corresponding to a pH = 7·35.

EXERCISE 78.

Electrometric Microanalysis of Calcium Oxide.

We will set ourselves the problem of estimating quantitatively an amount of CaO of the order of ·1 to ·2 milligram. In general this CaO would be precipitated in the form of oxalate from the

[1] The principle of this method was devised by Hasselbalch, *Biochem. Zeitschr.* **49**, 451. 1913, and is quoted from Michaelis, l.c.

solution of ash or other material under investigation. In this exercise, however, we will not trouble to prepare the oxalate in this way, but will start with some organic salt of calcium, and will convert this into CaO by heating in the same way as one would with the oxalate. The following procedure is to be adopted for the exact preparation of the small quantity of CaO that we wish to estimate.

A platinum crucible is heated and weighed. About ·15 to ·2 gm. of the purest powdered CaO (pro analysi, Kahlbaum) is weighed out in it and heated in the blowpipe until its weight is constant. In one particular experiment a quantity of ·1991 gm. was used. The CaO is then dissolved in 1 c.c. of diluted lactic acid, the process being facilitated by warming and by stirring with a glass rod, and the resulting solution is diluted to a volume of 400 c.c. with calcium-free distilled water. Each c.c. of this solution contains ·4978 mg. CaO. ·25 c.c. of it, containing ·1245 mg. of CaO, is measured into a platinum crucible, taken down to dryness on the water bath and then heated to redness for a few minutes. It is then placed for the time being in the desiccator.

We now prepare the following solution: 1 c.c. ·1 N. HCl + 5 c.c. 1 N. KCl solution + 44 c.c. of distilled water, i.e. a ·002 N. solution of HCl in ·1 N. KCl solution. 5 c.c. of this solution are measured out into the cooled platinum crucible and the contained CaO is brought completely into solution by suitable stirring and warming. The resulting solution is to be used for filling a pear-shaped electrode (fig. 30). The vessel is first washed with pure water and then with two successive 1 c.c. portions of the calcium solution, so that 3 c.c. remain over which are ample for the final filling. A second pear-shaped electrode is filled with the HCl solution not containing calcium. Both electrodes are filled with hydrogen in the way described on page 170 and then connected by means of saturated KCl solution. This concentration cell is placed in the compensation circuit in place of the standard cell, the calcium-free solution constituting the positive pole.[1]

At 16·5° C. the value obtained was ·0147 volt.

This potential difference is set up on account of the combination

[1] Or, according to the principle developed above, the two solutions can be measured successively in the same pear-shaped electrode against the saturated calomel electrode and the difference of the two readings taken as the value which the two solutions would give if directly opposed.

of some of the HCl with the CaO. The E.M.F. of the cell is therefore given by[1]

$$E = \cdot 0574 \log_{\cdot 10} \frac{h_1}{h_2}$$

where h_1 and h_2 represent the hydrogen ion concentrations of the calcium-free and of the calcium-containing solutions respectively. Since the addition of the KCl ensures that the total ionic concentration is almost identical in the two solutions the degree of dissociation[2] of the HCl will also be the same in both, so that the ratio $h_1 : h_2$ will be equal to that of the quantities of free HCl, $(HCl)_1 : (HCl)_2$, so that

$$\log. \frac{h_1}{h_2} = \log. \frac{(HCl)_1}{(HCl)_2} = \frac{\cdot 0147}{\cdot 0574} = \cdot 256,$$

and therefore

$$\frac{(HCl)_1}{(HCl)_2} = 1 \cdot 803,$$

or, since $(HCl)_1 = \cdot 002$ N.

$$(HCl)_2 = \cdot 00111 \text{ N.}$$

The 5 c.c. of ·002 N. HCl contained originally $5 \times \cdot 002 = \cdot 0100$ milli-equivalents of HCl. After the solution of the CaO $5 \times \cdot 00111 = \cdot 00555$ milli-equivalents of HCl remained unneutralised. The rest, ·00445 milli-equivalents having combined with an equal number of milli-equivalents of CaO. One milli-equivalent of CaO is 28 mgm. so that the total amount of CaO found is

$$\cdot 00445 \times 28 \text{ mgm.} = \cdot 1246 \text{ mgm. CaO.}$$

as compared with an expected value of ·1245 mgm.

So complete a correspondence is fortuitous. Errors of ± 5 per cent. of the total value may easily occur, and when the ratio of HCl to CaO is less favourable even greater discrepancies may be found. The most accurate results are obtained with quantities of calcium which give a potential difference between 8 and 50 millivolts, i.e. which neutralise from about a quarter to not more than three-quarters of the HCl present.

[1] The factor .0574 applies only to 16.5°C. For other temperatures the appropriate values should be taken from the table on p. 162.

[2] Or, preferably, in terms of Bjerrum's theory of ionic activities, we should say, instead of degree of dissociation the activity coefficient of the hydrogen ions.

EXERCISE 79.

Electrometric Titration.

The electrometric titration of an acid with a standard alkali enables one to follow step by step the changes of potential of the titration mixture against a hydrogen electrode and so the changes of hydrogen-ion concentration during the titration. The bell-shaped electrode shown in fig. 35 is used. The glass bell itself communicates above with a narrow glass tube provided with a tap. The platinum electrode consists of a wire fused through the roof of the bell and projecting a little below its mouth. The outer end of the wire passes into a fused-on side tube containing mercury, or, alternatively, is brought to a binding screw, in order to give an easy connection with the necessary copper lead. The electrode is supported as shown in fig. 35, so that its rim is just immersed in the fluid to be titrated which is contained in the dish. Into this solution there also dips a saturated KCl agar syphon (see p. 172), whose other end dips into a bath of saturated KCl, which is also in communication with a saturated calomel electrode (p. 173). The burette containing the standard alkali—·1 N. NaOH—is also arranged above the dish.

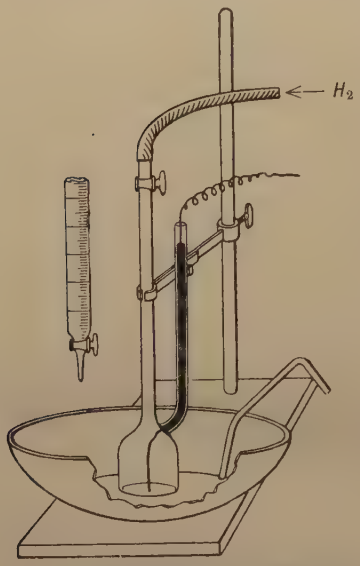

FIG. 35.—Electrometric Titration with Bell-shaped Electrode.

Before use the platinum wire is prepared in the following way. The projecting half centimeter or so of the wire is carefully heated to redness in a small flame and then the whole wire is wound into a loose spiral with the aid of a pair of forceps, so that it no longer projects but ends short of the edge of the bell by one or two millimeters. The whole electrode is now supported over a dish containing platinising solution and is filled with this by suction, and then the platinisation is carried out with the aid of a small

auxiliary platinum electrode immersed in the fluid outside (as on p. 169). The platinising solution is then run out and replaced by dilute sulphuric acid, and when the electrode has been cathodically polarised it is finally thoroughly washed.

Our first problem shall be to follow the titration of 10 c.c. of ·03 N. HCl (3 c.c. of ·1 N. HCl + 7 c.c. of water) with ·1 N. NaOH. The 10 c.c. of acid are placed in the porcelain dish and the alkali in the burette as depicted in fig. 35. A stream of hydrogen is now conducted through the bell at the rate of 1 to 2 bubbles a second until from $\frac{1}{2}$ to 1 litre of gas has passed. The tap is now turned off, and if the platinum wire is of the correct length it is not difficult to interrupt the gas stream at a moment when the tip of the wire is just immersed in the liquid. During the passage of the gas the wire should be alternately covered and uncovered as the bell slowly fills and then suddenly loses a bubble. This process provides a sufficient washing of the electrode by the solution after each addition of soda without further precaution. The smaller the area of platinum finally left immersed in the solution the more rapidly will a constant potential be established. The KCl-agar tube, which should have as fine a point as possible, is brought into the liquid and the potential is read off. The calomel electrode constitutes the positive pole. During the

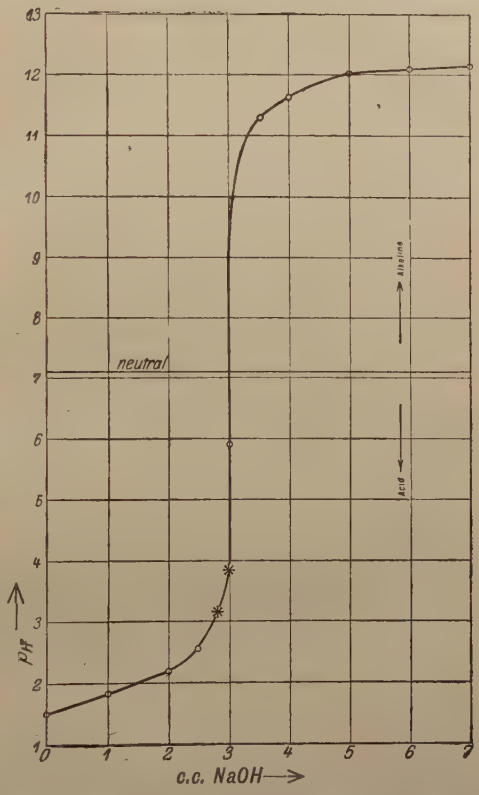

FIG. 36.—3 c.c. of ·1 N. HCl are treated with increasing quantities of ·1 N. NaOH. Abscissae, c.c. of soda solution. Ordinates pH.

establishment of the potential the apparatus must be guarded against shaking. It is sufficient if the reading is made to within 2–3 millivolts, it being in general unnecessary to wait for the exact establishment of the final equilibrium. The reading finished, ·5 c.c. of soda is run in, the liquid is mixed, and the passage of hydrogen recommenced. The establishment of the potential should occupy about 15 minutes, if it is not constant by this time it is a sign that the platinised surface has been spoiled. Further additions of soda are made in the same way, the whole titration requiring about 2 hours.

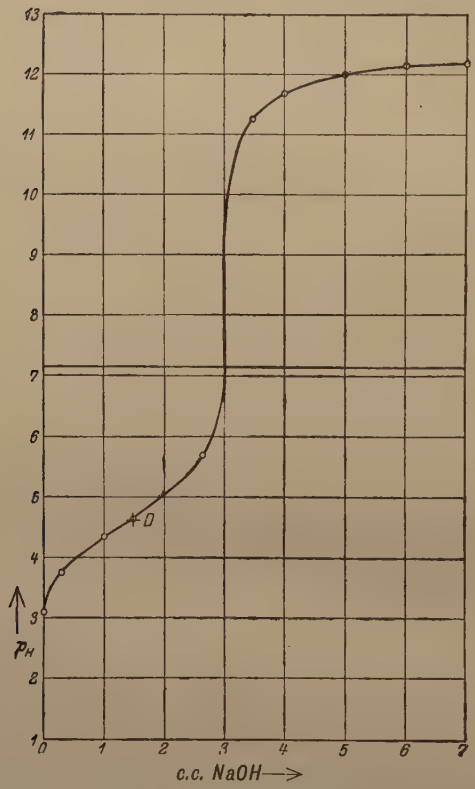

FIG. 37.—3 c.c. of ·1 N. acetic acid are titrated with ·1 N. NaOH. Co-ordinates as in Fig. 36. At D the value of the ordinate is equal to the negative logarithm of the ionisation constant of the acid that is being titrated.

The results are recorded graphically, c.c. of added soda being plotted as abscissae and millivolts, or, better still, the calculated pH values as ordinates. The result is given in the diagram of fig. 36. The circles are the points determined by experiment, and the curve is that calculated theoretically. The end point of the titration would be expected when 3 c.c. of soda have been added, and, as a matter of fact, at this point it is found that the pH jumps suddenly from 4 to 10—that is, from a value which is acid even to methyl orange to one which is alkaline even for phenolphthalein. This result affords the explanation for the well-known observation that it is of no consequence which of these two very dissimilar indicators is used for the titration of a mineral acid.

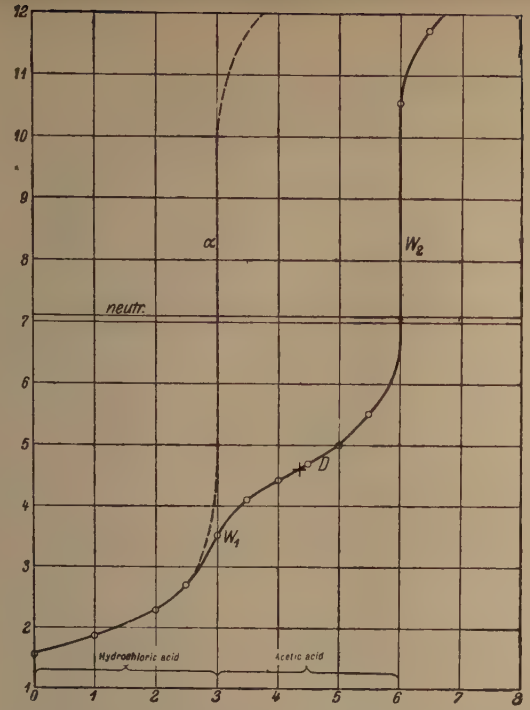

FIG. 38.—3 c.c. of ·1 N. HCl+3 c.c. of ·1 N. acetic acid titrated with ·1 N. NaOH. In the absence of the acetic acid the curve *a* would have been obtained. W_1 is the inflection marking the completion of the neutralisation of the HCl, W_2 is that marking the neutralisation of all the acetic acid. Further notes as in Figs. 36 and 37.

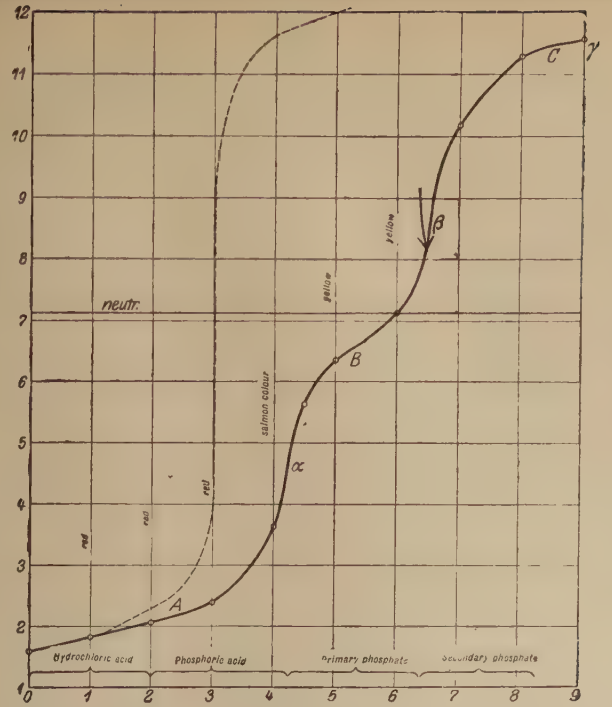

FIG. 39.—2 c.c. of ·1 N. HCl+2·2 c.c. of ·1 mol. phosphoric acid are titrated with ·1 N. NaOH. *a* is the sudden change of pH marking the complete formation of the primary phosphate and *β* is the corresponding inflection in the case of the secondary phosphate. The colour indications denote the tint that dimethylaminoazobenzene (see Exercise 22) would give. The arrow marks the turning point of phenolphthalein.

If acetic acid is titrated under identical conditions the curve shown in fig. 37 is obtained. The sudden change of pH that takes place when the equivalent amount of alkali has been added covers only the range from pH 7 to pH 10 only, and so is outside the range of methyl orange, and can be detected only by phenolphthalein.

If a mixture of 3 c.c. of ·1 N. HCl+3 c.c. of ·1 N. acetic acid is titrated (fig. 38) only a small inflection is observed at the point of neutralisation of the HCl, but the point of neutralisation of the acetic acid is marked by a second large inflection.

If a mixture of HCl and phosphoric acid is titrated (fig. 39) no characteristic inflection is found between the end of the neutralisation of the HCl and the beginning of that of the phosphoric acid, because these are both strong mineral acids. As soon as the formation of the primary phosphate is complete there is a jump from pH 4 to pH 5 (turning point of methyl orange), and as soon as the formation of the secondary salt is complete a second jump occurs from pH 8 to about pH 9·5 (turning point of phenolphthalein). The inflection that occurs during the formation of the tertiary phosphate is barely noticeable.

EXERCISE 80.

Membrane Potential and Donnan Equilibrium.[1]

If a membrane is interposed between two electrolytic solutions, and if the solution on one side of the membrane contains a "colloidal" ion that is incapable of diffusing through the membrane while the solution on the other side is free from such an ion, then the equilibrium which is established across the membrane between the two liquids is of a special kind. The presence of the colloidal ion that cannot pass through the membrane prevents the solutions from attaining ultimately the same composition. The condition of equilibrium that is actually set up is determined by the following principles:

1. The law of electric neutrality, according to which the sum of all the positive charges on the ions in a solution must be equal to the sum of all the negative ionic charges; this will apply to each of the two solutions individually. But the total sum of ionic charges must be greater in the solution containing the colloid than in the other solution. This law can be obeyed only if the colloidal ion

[1] F. G. Donnan, *Zeitschr. f. Elektrochemie*, **17**, 572, 1911.

expels an equivalent quantity of similarly charged diffusible ions from its solution, or attracts the equivalent quantity of oppositely charged ions into its solution or brings about a combination of these two processes in accordance with the law of equivalence.

2. In the equilibrium condition the ratio of the concentrations of the various diffusible cations present is the same for the two solutions, and, further, the ratio of the concentrations of the anions is the exact reciprocal of that of the cations.

3. If the ratio of the concentrations of any cation in the two solutions is $\gamma : 1$ there will be found between the two solutions a potential difference amounting to ·058 log. γ volt, i.e. a potential difference of the same magnitude as that given by a concentration

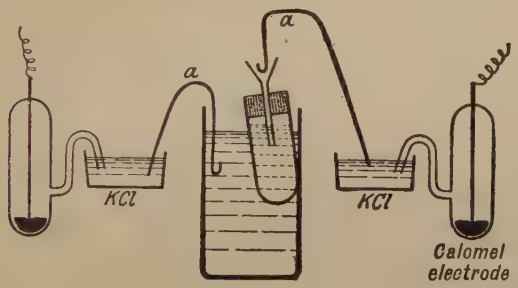

FIG. 40.

cell in which the ionic concentrations at the two electrodes are in the ratio $\gamma : 1$. We will now describe an experiment taken from Jacques Loeb, whose object is to demonstrate this fact.[1]

A collodion sac stuck to a rubber stopper (see pp. 97-98) and capable of holding about 40 c.c., is filled with a solution made by dissolving 1 gm. of gelatine in 100 c.c. of $^1/_{2000}$ N. HCl, and is then fitted with an almost capillary glass manometer tube about 20 cm. long. The sac is then immersed up to the rubber stopper in about 1 litre of $^1/_{2000}$ N. HCl contained in a large glass beaker, and is then left to itself for one or, better, two days in order that the diffusion equilibrium may be established. An osmotic pressure equal to that of a column of fluid of height 15–20 cms. will be set up. The manometer tube is now removed and replaced by a small funnel, and the potential difference between the two solutions is measured

[1] Jacques Loeb, *Proteins and the Theory of Colloidal Behaviour*, New York, 1922.

in the following way: By appropriately arranging the collodion sac so that it is lightly pressed against the bottom or side of the beaker a little of its contained fluid can be forced up into the funnel; into this fluid there is now immersed a KCl agar tube with bent point (see p. 128 and fig. 40, a), and this is connected electrically by means of a bath of saturated KCl solution to a saturated calomel electrode. A second agar tube similarly bent up at the point is immersed in the outer fluid and led off to a second calomel electrode. The E.M.F. between the two calomel electrodes is now measured in the usual way by the compensation method; a value of ·017 volt will be obtained, the gelatin solution forming the positive pole.

We now estimate by means of the H-ion concentration cell the pH of the inner and also of the outer solution. The result will be pH = 3·43 for the outer and pH = 3·76 for the inner solution, the difference being ·33, which corresponds to a potential difference in a H ion concentration cell made up with these two solutions of ·019 volt. The membrane potential has thus the same value, within the limits of experimental error, as is given by a H′-ion concentration cell made up with the inner and outer fluids; but at the same time it has the same value as would be given if the same solutions were used to make up a Cl′-ion concentration cell using electrodes such as mercury + calomel that are reversible with respect to Cl′ ions.

The difference between the total concentrations of ions and molecules in the two solutions determines the difference of osmotic pressure which shows itself in the manometer tube, and which may equally well be regarded as the " swelling pressure " of the gelatine.

XIV.

Chemical Kinetics.

In order to give an insight into the methods employed for the study of the kinetics of chemical reactions we will give the results of two actual experiments, together with the errors of observation, in order to enable us to decide which divergences from theory are inherent in the phenomena observed and which might possibly be expected from the magnitude of the experimental error.

EXERCISE 81.

The Hydrolysis of Cane Sugar by Acids.

Twenty gms. of cane sugar are dissolved in distilled water and made up to a volume of 200 c.c. The solution is placed in a 500 c.c. flask and immersed in a large water bath at room temperature. Into a second similar flask of the same size 200 c.c. of 4·0 N. HCl are measured out, and this is also placed in the water bath. During the warm summer months it is advisable to use acid of only half this strength. The temperature of the water bath is determined exactly, and is to be kept constant to within ·1° C. at least during the whole period of the experiment. When the temperature of the liquids has become uniform throughout the contents of the two flasks are mixed, the time is noted, and then as quickly as possible a sample is pipetted off into the polarimeter tube and its rotation is noted. Some 5 or 6 readings are taken quickly during the course of 1 to 2 minutes, the time of each individual reading being noted. The mean of the readings is then taken as the definitive value of the rotation at the mean point of the time interval over which the readings were spread. At intervals of 20 to 30 minutes further successive samples are withdrawn and examined in the same manner. The readings must be corrected for any error in the zero setting of the apparatus.

In a particular case the following values were obtained:

TABLE I.[1]

No.	Time in minutes. t	Rotation in °	Decrease of rotation x	$k_0 = x/t$
1.	0	[6·53]	0	—
2.	2	6·35	·18	·0900
3.	4·5	6·10	·43	·0955
4.	25·0	4·26	2·27	·0908
5.	41·5	3·05	3·48	·0838
6.	68·5	1·64	4·89	·0714
7.	98·5	·65	5·88	·0598
8.	128·5	−0·23	6·76	·0526
9.	149·5	−0·60	7·13	·0477
10.	24 hours	−2·19	8·72	—

The rotation of the fluid at the moment of mixing is obtained by extrapolation. Between readings 2 and 3 a change of rotation of ·25° has taken place. The rotation is therefore changing at the rate of ·10° per minute over this period. We therefore conclude that 2 minutes before the withdrawal of the second sample the rotation was ·20° higher than that of the second sample itself and take the initial value therefore as 6·55°. If we calculate the average rate of change between samples 2 and 4 we obtain a value of ·091° per minute, and using this figure for the extrapolation to zero time we obtain an initial reading of 6·53°. We cannot calculate the average rate of change of rotation from readings taken at wider time intervals than these, as this would involve the assumption that the rate of the reaction did not vary with time. But for short time intervals we can regard the rate of the reaction as proceeding with uniform velocity, and can place most reliance on the value of the initial rotation obtained by extrapolation from the results given by samples 2 and 4, particularly as it differs but little from that calculated from the observations on samples 2 and 3. We will thus take 6·53° as the initial value and place it in brackets in the table in order to indicate that it was calculated and not directly observed.

We wish now to determine the order of the reaction. We will start with the assumption that after the lapse of a sufficiently long

[1] For extraneous reasons these readings were made in a tube of length 18.94 cms. They are given as they were taken.

interval of time the inversion of the cane sugar would be complete, that is that the final equilibrium between cane sugar and invert sugar is such that there is only a vanishingly small concentration of cane sugar in the system. Under these circumstances the following possibilities arise:

1. A reaction of linear course or of "zero order." The amount of cane sugar disappearing at any instant is independent of the concentration of sugar present at that instant; throughout the course of the reaction equal quantities of sugar disappear during each equal interval of time.

$$x = k_0 \cdot t.$$

x denotes the amount of sugar that has disappeared at the time t, or, what comes to the same thing, the amount of invert sugar formed. k_0 is a proportionality factor which is independent of the initial concentration of cane sugar, but may vary with the temperature, the HCl concentration, and other factors. Such variations will not, however, be observed in our experiment, as we have kept the temperature and the HCl concentration constant throughout. If this expresses the conditions in the reacting system the relation must obtain that

$$\frac{x}{t} = k_0.$$

In this equation we may express x in any convenient units; our choice will influence merely the absolute value of k_0. We will thus express x by the total change of rotation that has occurred by the time t, this change being necessarily proportional to the amount of cane sugar that has disappeared and of invert sugar that has been formed. These values of x are given in the third column of Table I. The last column gives the values of k_0 for the various samples, and it will be seen that $\frac{x}{t}$ is not a constant, but decreases in value as the reaction proceeds. The reaction therefore does not follow a linear course.

2. A unimolecular reaction or reaction of the first order. The amount of sugar disappearing at each instant is a definite constant fraction of the total amount of sugar present at that instant. This condition is expressed by the differential equation:

$$\frac{dx}{dt} = k_1 \cdot (a - x),$$

where x as before denotes the amount of cane sugar that has disappeared (or of invert sugar that has been produced) after a time t, and a denotes the initial concentration of the cane sugar. On integration the equation becomes

$$\frac{1}{t} \cdot \log_{\cdot 10} \frac{a}{a-x} = \cdot 4343 \cdot k_1 = k.$$

In this expression, as contrasted with that for the linear reaction, the initial concentration a is involved, but only as the fraction $\frac{a}{a-x}$. It is therefore again a matter of indifference what unit we choose for expressing a and x We express them as before in degrees of change of rotation, but it is to be noted that a is the total completed change of rotation, i.e. not merely the initial rotation alone, but the sum of this with the amount of left-handed rotation at the end of the reaction thus

$$a = 6 \cdot 53 + 2 \cdot 19 = 8 \cdot 72.$$

If a cane sugar solution has an initial rotation m, after inversion the rotation will be ·31 to ·32 m. This factor varies to a considerable extent with the temperature and also with the concentration of the cane sugar. If we take ·32 as the basis of our calculation we obtain for the final rotation of our solution −2·09° a value which shows a satisfactory degree of correspondence with the observed value (−2·19°) when the above mentioned uncertainty in the value of the factor is taken into account. We will use the value actually observed in the experiment, and so calculate the values in Table II.

TABLE II.

$a = 8 \cdot 72.$ log. $a = \cdot 941.$

t	x	$a-x$	log. $(a-x)$	log. $\frac{a}{a-x}$	$\frac{1}{t} \cdot \log. \frac{a}{a-x} = k$
0	0	8·72	·941	—	—
2	·18	8·54	·931	·010	·00500
4·5	·43	8·29	·919	·022	·00488
25·0	2·27	6·45	·810	·131	·00524
41·5	3·48	5·24	·719	·222	·00535
68·5	4·89	3·83	·583	·358	·00523
98·5	5·88	2·84	·453	·488	·00500
128·5	6·76	1·96	·292	·649	·00503
149·5	7·13	1·59	·201	·740	·00495

Mean value, $k_m = \cdot 00508$

It will be seen that the values in the last column are sensibly constant so that we may say that the law of a unimolecular reaction is obeyed at least approximately. In order to decide this point yet more exactly we proceed as follows: We take the arithmetical mean of the various values of k; this is denoted by k_m in the table, and has a value of ·00508. Using this value we calculate for each value of t the corresponding theoretical value of $(a-x)$, transforming our fundamental equation for convenience as follows:

$$\log.\ (a-x) = \log.\ a - kt.$$

The results of this calculation are given in Table III.

TABLE III.

$k_m = ·00508.$ log. $a = ·941.$

t	$k_m \cdot t$	log. $a - k_m t$ = log. $(a-x)$	$a-x$ calculated	$a-x$ observed	Difference between calculated and observed values of $a-x$ in degrees
0	—	·941	[8·72]	8·72	—
2·0	·0102	·931	8·53	8·54	+·01
4·5	·0229	·918	8·28	8·29	+·01
25·0	·127	·814	6·52	6·45	−·07
41·5	·218	·723	5·29	5·24	−·05
68·5	·348	·593	3·92	3·83	−·09
98·5	·500	·441	2·76	2·84	+·08
128·5	·658	·288	1·94	1·96	+·02
149·5	·760	·181	1·52	1·59	+·07

The difference between observed and calculated values in no case exceeds a few hundredths of a degree, and so always falls within the limits of experimental error. The close agreement of the values for the 2 and 4·5 minute periods is purely accidental. On account of the small amount of change that has taken place and the smallness of the time interval to be measured a considerably greater degree of divergence would have been permissible.

If these two uncertain initial values are neglected the remaining values of the constant seem to show a slight continuous drift inasmuch as the differences are not distributed indiscriminately about a mean value, but are at first all negative, and then for the later points become positive. The divergences are admittedly small, but still, nevertheless, this gradual change is well marked. Are we therefore justified in ascribing an actual significance to it?

This is not the case. The value we used for a was obtained by extrapolation, and therefore involves a somewhat greater element of uncertainty than the other numbers. If we take a value for a only ·02° smaller—and such a variation might easily fall within the limits of experimental error—the whole calculation would be affected in such a way as to eliminate completely any drift in the values obtained for the constant, as a re-calculation would readily show.

EXERCISE 82.

The Fermentative Splitting of Cane Sugar.

As ferment we will use the saccharase (invertin, invertase) of yeast. 100 gms. of fresh bakers' yeast are stirred in a mortar with a little and then with more and more water until the volume of the suspension amounts to 200 c.c. Five c.c. of chloroform are then added, and the liquid is placed in an incubator in a closed vessel for 4 to 5 days. The material is then acidified by the addition of a few drops of strong acetic acid, about 20 gms. of finely powdered kaolin is added with thorough shaking, and then after a few minutes the mixture is filtered through a large folded filter. The first portion of the filtrate often comes through milky, in which case it is returned to the stock flask. The filtrate is only collected for use when it forms a slightly yellow but quite clear solution. For our purpose a further purification by dialysis is unnecessary. The ferment may be kept practically indefinitely without loss of activity if a little chloroform or toluol is added and it is stored in the ice-chest. Its activity will naturally vary somewhat according to the properties of the yeast from which it was obtained.

A water bath fitted with a stirrer is now required. It must be maintained at a temperature of 25° C. with a maximum variation of $\pm$·05° C.

A solution of about 10 gms. of cane sugar in 300 c.c. of distilled water is made up in a 500 c.c. flask and placed to warm in the water bath. In a similar flask a mixture of 20 c.c. of the ferment solution + 20 c.c. of ·1 N. sodium acetate + 20 c.c. of ·1 N. acetic acid + 140 c.c. of distilled water, making 200 c.c. in all; this is also set in the bath. Meanwhile 10 to 12 Erlenmeyer flasks are prepared outside the bath, each containing exactly 3 c.c. of ·5 mol. Na_2CO_3. The strength of this sodium carbonate solution is so chosen that when the acid sugar solution is added to it in the way

about to be described the mixture shall be distinctly alkaline to litmus.

When both flasks have been brought to the temperature of the bath their contents are mixed, the time is noted, and then as quickly as possible a 25 c.c. portion is pipetted off and run into one of the flasks of sodium carbonate solution. The time at which this first sample is withdrawn is noted. At suitable intervals further successive samples are withdrawn in the same way.

The object of the alkali is two-fold. In the first place it stops the action of the enzyme; secondly, it ensures that the multirotation of the glucose freshly liberated by the invertase shall be brought at once to the normal value. In the case of the hydrolysis by acid the definitive rotation is at once established, the addition of alkali was therefore not necessary in the previous experiment. The acidification of the ferment solution by means of the acetate mixture has for object the provision of the most favourable hydrogen ion concentration for the activity of the invertase.

The individual samples are examined by means of the polarimeter. The last sample should be taken at the end of 24 hours. The final value often fails to coincide with the theoretical end-point of the reaction because the end stages of the hydrolysis proceed very slowly.

The end value to be expected from complete hydrolysis can be calculated as follows: If the initial rotation is $+ a$ and the theoretical end reading $- b$, then $b = \cdot 313 \times a$. The total range of change of the rotation is therefore $1 \cdot 313 \times a$.

It is advisable to determine the rotation of the ferment itself in a control mixture made up without the sugar and to introduce this value as a correction into all the readings; but in any case this correction will be very small.

The results of an actual experiment were as follows:

Times in minutes t	Corrected rotation	Change of rotation	$\frac{x}{t} = k_0$	$\frac{1}{t} \cdot \log. \frac{a}{a-x} = k$
0	[4·334]	0	—	—
·5	4·324	·010	—	—
21·0	3·945	·389	·0185	·00145
60·0	3·260	1·074	·0179	·00151
130·0	2·129	2·205	·0170	·00164
190·2	1·330	3·004	·0158	·00171
246·0	·744	3·590	·0146	·00176

As before the relative quantities of sugar are expressed in terms of degrees of rotation.

We will now determine the order of the reaction. Column 4 of the above table gives the calculated values of k_0 on the assumption that the reaction follows a linear course. But these values of k_o show a continuous drift; they fall off as the reaction proceeds.

Column 5 gives the values of the constant $k = \frac{1}{t} \cdot \log. \frac{a}{a-x}$ calculated for a reaction of the first order.

The value of a is equal to the total theoretically possible attainable change of rotation, and is given by $4{\cdot}334\ (1 + {\cdot}313) = 5{\cdot}690$.

In this case also the values of k show a decided drift, but now they increase with time. In order to determine whether the magnitude of this drift falls within the limits of experimental error we calculate the mean value of k, and using this value, $k_m = {\cdot}001614$, we calculate backwards the successive values of $a-x$. The result is as follows:

t	$a-x$ calculated	$a-x$ observed	Difference between calculated and observed values.
0	—	5·690	—
21·5	5·167	5·301	+·134
60·0	4·489	4·616	+·127
130·0	3·461	3·485	+·024
190·2	2·766	2·686	−·080
246·0	2·248	2·100	−·148

The differences show a marked drift, and particularly at the beginning and the end of the experiment are distinctly greater than the expected limits of experimental error. That the differences are small round about the mid-point of the experiment is due to the circumstance that a mean value of k was used as the basis of the calculation. The progress of the fermentative splitting of cane sugar does not follow the law of a unimolecular reaction. Its course is intermediate between that of a linear and that of a logarithmic process; it is "infra-logarithmic." But the explanation of the significance of such a course and the elaboration of the formula that is necessary for its accurate expression are beyond the scope of this work.

TABLE OF LOGARITHMS.

See p. 29.

	0	0·5	1	1·5	2	2·5	3	3·5	4	4·5	5	6	7	8	9
1	000	021	041	061	079	097	114	130	146	161	176	204	230	255	279
		979	*959*	*939*	*921*	*903*	*886*	*870*	*854*	*839*	*824*	*796*	*770*	*745*	*721*
2	301	312	322	332	342	352	362	371	386	389	398	416	431	447	462
	699	*688*	*678*	*668*	*658*	*648*	*638*	*629*	*620*	*611*	*602*	*585*	*569*	*553*	*538*
3	477	484	491	498	505	512	519	525	531	538	544	556	568	580	591
	523	*516*	*509*	*502*	*495*	*488*	*481*	*475*	*469*	*462*	*456*	*444*	*432*	*420*	*409*
4	602	607	613	618	623	628	633	638	643	648	653	663	672	681	690
	398	*393*	*387*	*382*	*377*	*372*	*367*	*362*	*357*	*352*	*347*	*337*	*328*	*319*	*310*
5	699	703	708	712	716	720	724	728	732	737	740	748	756	763	771
	301	*297*	*292*	*288*	*284*	*280*	*276*	*272*	*268*	*263*	*260*	*252*	*244*	*237*	*229*
6	778	782	785	789	792	796	799	803	806	810	813	820	826	833	839
	222	*218*	*215*	*211*	*208*	*204*	*201*	*197*	*194*	*190*	*187*	*180*	*174*	*167*	*161*
7	845	848	851	854	857	860	863	866	869	872	875	881	886	892	898
	155	*152*	*149*	*146*	*143*	*140*	*137*	*134*	*131*	*128*	*125*	*119*	*114*	*108*	*102*
8	903	906	908	911	914	916	919	922	924	927	929	935	940	944	949
	097	*094*	*092*	*089*	*086*	*084*	*081*	*078*	*076*	*073*	*071*	*065*	*060*	*056*	*051*
9	954	957	959	961	964	966	968	971	973	975	978	982	987	991	996
	046	*043*	*041*	*039*	*036*	*034*	*032*	*029*	*027*	*025*	*022*	*018*	*013*	*009*	*004*

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